Supporting Information for:

Caged Phosphoproteins

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General Experimental Procedures

All starting amino acids and reagents used are commercially available, unless referenced to synthetic procedure. Dichloromethane was distilled from calcium hydride under nitrogen, and tetrahydrofuran was distilled from sodium under argon. Analytical thin-layer chromatography (TLC) was carried out on F_{254} 250-μm silica gel plates, and visualized by UV lamp. ^{1}H NMR spectra were acquired on a Bruker Avance (DPX) 400 MHz spectrometer or Varian Mercury 300 MHz spectrometer. ^{31}P NMR spectra were acquired on a Varian Mercury 300 MHz spectrometer. ^{13}C NMR spectra were acquired on a Varian Inova 500 MHz spectrometer or a Bruker Avance (DPX) 400 MHz spectrometer. Chemical shifts are reported in ppm from a standard (tetramethylsilane for ^{1}H, H_2PO_4 for ^{31}P, $CDCl_3$ for ^{13}C), and *J* values are in Hertz. High-resolution mass spectrometry was performed on a Fourier Transform Mass Spectrometer using an Electrospray Ion Source. High-performance liquid chromatography (HPLC) was performed using a Waters 600E HPLC fitted with a Waters 600 automated control module and a Waters 2487 dual wavelength absorbance detector recording at 228 and 280 nm. For analytical HPLC a Beckman Ultrasphere C_{18}, 5 μm, 4.6 x 150 mm reverse-phase column was used. For preparative separations a YMC-pack, C_{18}, 250 x 20 mm reversed phase column was used. The standard gradient for analytical and preparatory HPLC used was 93:7 to 0:100 over 35 minutes (water:acetonitrile, 0.1% TFA). Electrospray Ionization Mass Spectrometry (ESI-MS) was performed on a PerSeptive Biosystems *Mariner ™ Biospectrometry Workstation* (Turbo Ion Source). All tRNAs were analyzed on a PerSeptive Biosystems (Framingham, MA) Voyager DE PRO MALDI-TOF mass spectrometer operating in linear and positive ion modes with settings as previously described.[1]

Chemical Synthesis

***O*-1-(2-nitrophenyl)ethyl-*O'*-tertbutyl-*N,N*-diethyl phosphoramidite (3).** To an oven dried flask with stir bar, hexaethyl phosphorus triamide (1.37 mL, 5.00 mmole) and 4,5-dicyanoimidazole (0.58 g, 4.90 mmole) are dissolved in anhydrous tetrahydrofuran (THF, 15 mL) under inert atmosphere. In an oven dried pear flask, *tert*-Butyl alcohol (0.48 mL, 5.00 mmole) was dissolved in anhydrous THF (5.0 mL). In a separate oven dried pear flask, 1-2-(nitrophenyl)ethyl alcohol (1.00 g, 6.00 mmole) was dissolved in anhydrous THF (1 mL). The *t*BuOH solution was delivered to the phosphoramidite mixture via cannula, and the reaction allowed to stir at room temperature for 15 minutes, at which point, the nitrophenylethyl alcohol mixture was delivered to the reaction flask via cannula. The mixture was allowed to stir overnight, in the dark, at room temperature. The mixture was then concentrated under reduced pressure, and re-dissolved in ethyl acetate (EtOAc, 150 mL). The organic solution was washed with 20 % sodium bicarbonate ($NaHCO_3$, 2 x 150 mL) and brine (1 x 150 mL), then dried over magnesium sulfate ($MgSO_4$), filtered, and concentrated. The final product was purified by silica gel flash chromatography (98:2 Hexanes/triethylamine (NEt_3), R_f: 0.73,) to give **5** (1.27 g) in 74.0 % yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.92 (td, J_{HH} = 1.8 Hz, J_{HH} = 8.4 Hz, 2H), 7.65 (tt, J_{HH} = 1.5 Hz, J_{HH} = 7.5, 1H), 7.40, (tt, J_{HH} = 1.5 Hz, J_{HH} = 8.4 Hz, 1 H), 5.55 (m, 1 H), 3.06 (m, 4H), 1.57 (dd, J_{HH} = 4.5 Hz, J_{HH} = 6.3 Hz, 3H), 1.30 (d, J_{HH} = 19.5 Hz, 9H), 1.08 (dt, J_{HH} = 6.0 Hz, J_{HH} =43.5 Hz, 6 H). ^{31}P NMR (121.5 MHz, $CDCl_3$) δ ppm: 139.93, 139.16 (racemic mixture). ^{13}C NMR (125.8 MHz, $CDCl_3$) 147.9, 142.0, 133.8, 129.6, 128.2, 124.5, 75.5, 67.0, 38.3, 31.3, 25.9, 15.6. Product is not stable to MS measurements.

***N*$^{\alpha}$-4-pentenoyl-*O-tert*-butyl-L-Serine:** H-*O-tert*-butyl-L-Serine (**1a**, 500 mg, 3.10 mmole) and *N,N*-diisopropylethylamine (DIPEA, 633 μL, 3.70 mmole) were dissolved in THF (30 mL) and water (30 mL). 4-pentenoic anhydride (676 μL, 3.70 mmole) was dissolved in THF (2 mL), and added to the serine solution. The reaction was monitored by TLC (1:1:0.01 hexanes/EtOAc/acetic acid (AcOH), R_f = 0.30), stained with iodine and ninhydrin. After the disappearance of starting material, the THF was removed under reduced pressure, and the water layer extracted with EtOAc (3 x 100 mL). The organic portions were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The product was purified by silica gel flash chromatography (827 mg) in 93.7 % yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 6.99 (d, J_{HH} = 8.1 Hz, 1 H), 5.70 (m, 1 H), 4.94 (q, J_{HH} = 17.1, 10.2, 10.2 Hz, 2 H), 4.64 (m, 1 H), 3.71 (dd, J_{HH} = 9 Hz, J_{HH} = 3 Hz, 1 H), 3.46 (dd, J_{HH} = 9.3 Hz, J_{HH} =3 Hz, 1 H), 2.25 (s, 4 H), 0.99 (s, 9 H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 176.0, 173.9, 136.7, 115.8, 73.7, 61.9, 53.0, 35.3, 29.7, 27.3. ESI-MS: $[M-H]^-$ 242.1401 (obsd), 242.1398 (calcd).

***N*$^{\alpha}$-4-pentenoyl-*O-tert*-butyl-L-Serine cyanomethyl ester:** *N* $^{\alpha}$-4-pentenoyl-*O-tert*-butyl-L-Serine (825 mg, 3.39 mmole) was dissolved in chloroacetonitrile (644 μL, 10.20 mmole), and cooled to 0°C, stirring, under argon. Diazabicyclo[5.4.0]undec-7-ene (DBU, 507 μL, 3.39 mmole) was slowly added to the stirring mixture. The reaction was allowed to warm to room temperature, and stir overnight under argon. The reaction mixture was diluted with EtOAc (150 mL), washed with water (2 x 150 mL), then brine (1 x 150 mL), dried over $MgSO_4$, filtered and concentrated (1:1 hexanes/EtOAc, R_f = 0.41) (765 mg, 79.9 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 6.81 (d, J_{HH} = 8.7 Hz, 1 H), 5.83 (m, 1 H), 5.11 (q, J_{HH} = 18.3, 6.6, 9.9 Hz, 2 H), 4.84 (m, 3 H), 3.86 (dd, J_{HH} = 9.3 Hz, J_{HH} = 3.3 Hz, 1 H), 3.58 (dd, J_{HH} = 9.3 Hz, J_{HH} = 3.3, 1 H), 2.38 (s, 4 H), 1.16 (s, 9 H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 172.7, 169.6, 137.1, 115.7, 114.5, 73.8, 61.9, 52.7, 49.2, 35.2, 29.5, 27.3. ESI-MS: $[MNa]^+$ 305.1478 (obsd), 305.1472 (calcd).

***N*$^{\alpha}$-4-pentenoyl-L-Serine cyanomethyl ester (2a):** *N*$^{\alpha}$-4-pentenoyl-*O-tert*-butyl-L-Serine cyanomethyl ester (750 mg, 2.69 mmole) was dissolved in an ice-cold solution of triflouroacetic acid (TFA) with 1 % (v/v) triisopropylsilane (TIS). The reaction was stirred at 0°C for 4 hours, and determined complete by TLC (EtOAc, R_f = 0.43). The reaction mixture was poured into ice-cold saturated $NaHCO_3$ (250 mL) and EtOAc (120 mL). The organic layer was washed with saturated $NaHCO_3$ (3 x 100 mL), then brine (1 x 100 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The mixture was purified by silica gel flash chromatography to give the product (156 mg) in 25.6 % yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 6.90 (d, J_{HH} = 7.8 Hz, 1 H), 5.87 (m, 1 H), 5.10 (t, J_{HH} = 19.5, 10.8 Hz, 2 H), 4.80 (d, J_{HH} = 1.5 Hz, 3 H), 4.70 (m, 1 H), 4.06 (dd, J_{HH} = 11.7 Hz, J_{HH} = 3.6, 3.3 Hz, 1 H), 3.87 (dd, J_{HH} = 11.4 Hz, J_{HH} = 3.6, 3.6 Hz, 1 H), 2.38 (d, J_{HH} = 3.0 Hz, 4 H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 173.5, 169.8, 137.0, 116.4, 114.5, 63.0, 54.6, 49.7, 35.8, 29.8. ESI-MS: $[MNa]^+$ 249.0847 (obsd), 249.0846 (calcd).

N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl-2-*tert*-butyl)-L-Serine cyanomethyl ester (4a): N^{α}-4-pentenoyl-L-Serine cyanomethyl ester (**2a**, 150 mg, 663 μmole) was dissolved in anhydrous THF (2.0 mL), in a round bottom flask fitted with an oven dried stir bar under argon. In a separate pear flask, *O*-1-(2-nitrophenyl)ethyl-*O'-tert*butyl-*N,N*-diethyl phosphoramidite (**3**, 340 mg, 995 μmole) and 4,5-dicyanoimidazole (157 mg, 1.33 mmole) were dissolved in anhydrous THF (1.3 mL), and kept dark. The phosphoramidite solution was delivered to the serine solution via syringe, and the reaction was allowed to stir overnight, under argon, in the dark. The reaction was judged complete by loss of starting material by TLC. The mixture was concentrated under reduced pressure, and redissolved in EtOAc (50 mL). The organic layer was washed with 10 % $NaHCO_3$ (2 x 80 mL), brine (1 x 80 mL), then dried over sodium sulfate (Na_2SO_4), decantend and concentrated. The phosphite mixture was then oxidized with *tert*-butyl hydroperoxide (*t*BuOOH, 266 μL of 5 M solution in decane, 1.33 mmole) in dichloromethane (CH_2Cl_2, 10 mL) for one hour, in the dark. The mixture was diluted with CH_2Cl_2 (50 mL), and washed with 10 % $NaHCO_3$ (2 x 80 mL) and brine (1 x 80 mL), then dried over $MgSO_4$, filtered and concentrated. The product was partially purified by silica gel flash chromatography (1:2 hex/EtOAc, R_f = 0.26), and carried on to the next step before final purification (68.2 %).

N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl)-L-Serine cyanomethyl ester (5a): N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl-2-*tert*-butyl)-L-Serine cyanomethyl ester (**4a**, 69 mg, 135 μmole) was dissolved in acetonitrile (MeCN, 4.0 mL) at room temperature, stirring, and kept dark. A solution of TFA (1.0 mL) with TIS (50 μL) was added to the solution, and stirred for 10 minutes at room temperature. The mixture was then poured into ice-cold saturated $NaHCO_3$ (4.0 mL). The product mixture was purified by reversed phase HPLC. (93:7 to 0:100 water/MeCN/0.1 % TFA, over 35 minutes, R_t = 22.68 min) in 99 % yield (61.0 mg). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.00 (ddd, J_{HH} = 8.1 Hz, J_{HH} = 3.6, 3.6 Hz, J_{HH} = 1.5, 1.2, 1.2, 1.5 Hz, 1 H), 7.79 (m, 2 H), 7.53 (m, 1 H), 6.04 (m, 1 H), 5.86 (m, 1 H), 5.11 (m, 2 H), 4.88 (m, 3 H), 4.40 (m, 3 H), 2.40 (d, J_{HH} = 7.5 Hz, 4 H), 1.71 (dd, J_{HH} = 6.3 Hz, J_{HH} = 1.2, 1.2 Hz, 3 H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 173.7, 168.3, 147.3, 137.5, 137.0, 134.6, 129.5, 128.2, 125.0, 116.3, 114.2, 73.3, 67.2, 52.8, 50.1, 35.5, 29.7, 24.7. ^{31}P (121.5 MHz, $CDCl_3$): -1.382. ESI-MS: $[M-H]^-$ 454.1017 (obsd), 454.1021 (calcd).

N^{α}-4-pentenoyl-*O-tert*-butyl-L-Threonine: H-*O-tert*-butyl-L-Threonine (**1b**, 500 mg, 2.85 mmole) and DIPEA (683 μL, 3.99 mmole) were dissolved in THF (40 mL) and water (10 mL). 4-pentenoic anhydride (730 μL, 3.99 mmole) was dissolved in THF (5 mL), and added to the threonine solution. The reaction was monitored by TLC (1:1:0.01 hexanes/EtOAc/AcOH, R_f = 0.31), stained with iodine and ninhydrin. After the disappearance of starting material, the mixture was concentrated under reduced pressure, and the product was purified by silica gel flash chromatography (625 mg, 85.3 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 6.74 (d, J_{HH} = 8.4 Hz, 1 H), 5.88 (m, 1 H), 5.12 (q, J_{HH} = 17.4, 8.1, 10.2 Hz, 2 H), 4.60 (dd, J_{HH} = 8.7 Hz, J_{HH} = 2.7, 2.4 Hz, 1 H), 4.32 (m, 1 H), 2.43 (m, 4 H), 1.20 (s, 9 H), 1.71 (d, J_{HH} = 6.6 Hz, 3 H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 174.3, 174.0, 136.9, 116.0, 75.2, 67.2, 57.7, 35.6, 29.7, 28.4, 20.1. ESI-MS: $[M-H]^-$ 256.1551 (obsd), 256.1554 (calcd).

N^{α}-4-pentenoyl-*O-tert*-butyl-L-Threonine cyanomethyl ester: N^{α}-4-pentenoyl-*O-tert*-butyl-L-Threonine (390 mg, 1.51 mmole) was dissolved in chloroacetonitrile (478 μL, 7.55 mmole) and cooled to 0°C, stirring, under argon. DBU (226 μL, 1.51 mmole) was slowly added to the stirring mixture. The reaction was allowed to warm to room temperature, and stir overnight under argon. The reaction mixture was diluted with EtOAc (100 mL), washed with water (2 x 100 mL) and brine (1 x 100 mL), then dried over $MgSO_4$, filtered and concentrated (1:1 hexanes/EtOAc, R_f = 0.43) (321 mg, 71.7 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 6.39 (d, J_{HH} = 9.0 Hz, 1 H), 5.91 (m, 1 H), 5.14 (q, J_{HH} = 17.1, 7.5, 10.2 Hz, 2 H), 4.78 (d, J_{HH} = 3.0 Hz, 2 H), 4.61 (dd, J_{HH} = 9.3 Hz, J_{HH} = 2.1, 1.8 Hz, 1 H), 4.27 (m, 1 H), 2.42 (d, J_{HH} = 2.7 Hz, 4 H), 1.21 (d, J_{HH} = 6.3 Hz, 3 H), 1.14 (s, 9H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 173.1, 169.9, 137.1, 115.9, 114.3, 74.5, 67.2, 57.7, 49.1, 35.5, 29.5, 28.6, 21.0. ESI-MS: $[MNa]^+$ 319.1617 (obsd), 319.1628 (calcd).

N^{α}-4-pentenoyl-L-Threonine cyanomethyl ester (2b): N^{α}-4-pentenoyl-*O-tert*-butyl-L-Threonine cyanomethyl ester (315 mg, 1.06 mmole) was dissolved in an ice-cold solution of TFA with 1 % (v/v) TIS. The reaction was stirred at 0°C for two hours, and determined complete by TLC (1:1 hex/EtOAc, R_f = 0.15). The reaction mixture was poured into ice-cold saturated $NaHCO_3$ (80 mL) and EtOAc (80 mL). The organic layer was washed with saturated $NaHCO_3$ (2 x 80 mL) and brine (1 x 80 mL), then dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The mixture was purified by silica gel flash chromatography to give the product (150 mg) in 59.0 % yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 6.39 (d, J_{HH} = 8.4 Hz, 1 H), 5.90 (m, 1 H), 5.14 (t, J_{HH} = 17.4, 13.8 Hz,

2 H), 4.87 (q, J_{HH} = 15.6, 7.5, 15.6 Hz, 2 H), 4.69 (dd, J_{HH} = 9.0 Hz, J_{HH} = 2.7, 2.4 Hz, 1 H), 4.46 (qd, J_{HH} = 6.3, 6.3, 6.3 Hz, J_{HH} = 2.4, 2.4, 2.4, 2.4 Hz, 1 H), 2.43 (d, J_{HH} = 2.7 Hz, 4 H), 1.27 (t, J_{HH} = 4.2, 2.1 Hz, 3 H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 173.7, 170.2, 137.1, 116.4, 114.5, 68.0, 57.5, 49.6, 36.0, 29.8, 20.6. ESI-MS: $[MNa]^+$ 263.1005 (obsd), 263.1002 (calcd).

N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl-2-*tert*-butyl)-L-Threonine cyanomethyl ester (4b): N^{α}-4-pentenoyl-L-Threonine cyanomethyl ester (140 mg, 582 μmole) was dissolved in anhydrous THF (2 mL), in a round bottom flask fitted with an oven dried stir bar under argon. In a separate pear flask, *O*-1-(2-nitrophenyl)ethyl-*O'*-*tert*butyl-*N,N*-diethyl phosphoramidite (**3**, 299 mg, 874 μmole) and 4,5-dicyanoimidazole (137 mg, 1.16 mmole) were dissolved in anhydrous THF (1 mL), and kept dark. The phosphoramidite solution was delivered to the threonine solution via syringe, and the reaction was allowed to stir overnight, under argon, in the dark. The reaction was judged complete by loss of starting material by TLC. The mixture was concentrated under reduced pressure, and re-dissolved in EtOAc (80 mL). The organic layer was washed with 10 % $NaHCO_3$ (2 x 80 mL), brine (1 x 80 mL), then dried over Na_2SO_4, decantend and concentrated. The phosphite mixture was then oxidized with *t*BuOOH (232 μL of 5 M solution in decane, 1.16 mmole) in dichloromethane (CH_2Cl_2, 10 mL) for one hour, in the dark. The mixture was diluted with CH_2Cl_2 (70 mL), and washed with 10 % $NaHCO_3$ (2 x 80 mL), brine (1 x 80 mL), then dried over $MgSO_4$, filtered and concentrated The product was partially purified by silica gel flash chromatography (R_f = 0.19, 1:1 hex/EtOAc) and carried on to the next step before final purification (70.1 %).

N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl)-L-Threonine cyanomethyl ester (5b): N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl-2-*tert*-butyl)-L-Threonine cyanomethyl ester (250 mg, 476 μmole) was dissolved in MeCN (3.5 mL) at room temperature, stirring, and kept dark. A solution of TFA (3.5 mL) with TIS (70 μL) was added to the solution, and stirred for 15 minutes at room temperature. The mixture was then poured into ice-cold saturated $NaHCO_3$ (10 mL). The mixture was purified by reversed phase HPLC (93:7 to 0:100 water/acetonitrile/0.1 % TFA over 35 minutes, R_t = 23.86 min) in 41.0 % yield (92.0 mg). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.00 (d, J_{HH} = 7,=.8 Hz, 1 H), 7.79 (m, 2 H), 7.55 (m, 1 H), 6.89 (t, J_{HH} = 7.2, 9.0 Hz, 1 H), 6.53 (bs, 1 H), 6.05 (m, 1 H), 5.87 (m, 1 H), 5.13 (m, 2 H), 4.92 (m, 6 H), 2.42 (d, J_{HH} = 2.1 Hz, 4 H), 1.74 (d, J_{HH} = 6.3 Hz, 3 H), 1.35 (dd, J_{HH} = 24.0 Hz, J_{HH} = 6.3, 6.6 Hz, 3 H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 174.8, 169.1, 147.8, 137.6, 137.0, 134.7, 129.8, 128.5, 125.2, 116.8, 114.5, 75.8, 73.7, 56.6, 50.5, 35.9, 30.1, 24.9, 19.2. ^{31}P (121.5 MHz, $CDCl_3$): -1.13, -1.17. ESI-MS: $[M-H]^-$ 468.1168 (obsd), 468.1177 (calcd).

N^{α}-4-pentenoyl-L-Tyrosine *tert*-butyl ester: H-L-Tyrosine-*tert*-butyl ester (475 mg, 2.0 mmol) was dissolved in anhydrous THF (15 mL) and anhydrous CH_2Cl_2 (5 mL). DIPEA (975 μL, 6.0 mmol) was added, and pentenoic anhydride (440 μL, 2.4 mmol) was added dropwise to the resulting mixture. The reaction mixture was stirred at room temperature for 45 minutes and then concentrated under reduced pressure. The clear oil was purified by silica gel flash chromatography (R_f = 0.70, 2:3 hex/EtOAc) to give the desired product as a clear oil (568 mg, 89%). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 1.42 (s, 9H), 2.0-2.3 (m, 4H), 2.9-3.0 (m, 2H), 4.71 (dd, 1H, J_1 = 7.6 Hz, J_{HH} = 6.2 Hz), 4.94-5.01 (m, 2H), 5.67-5.77 (m, 1H), 6.42 (d, 1H, J_{HH} = 7.9 Hz), 6.77 (d, 2H, J_{HH} = 8.3 Hz), 6.97 (d, 2H, J_{HH} = 8.3 Hz), 8.28 (s, 1H). ^{13}C-NMR (100.6 MHz, $CDCl_3$, δ): 172.9, 171.8, 155.8, 136.5, 130.4, 126.8, 115.9, 115.5, 82.6, 53.9, 37.2, 35.6, 29.4, 27.8. ESI-MS: $[M+H]^+$ 320.1855 (obsd), 320.1862 (calcd).

N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-Tyrosine *tert*-butyl ester: N^{α}-4-pentenoyl-L-Tyrosine *tert*-butyl ester (1.13 g, 3.55 mmol) was placed in an oven dried round bottom flask with molecular sieves and dissolved in anhydrous THF (10 mL). In a separate flask, *N,N*-Diisopropyl-1-nitrophenylethyl-2-cyanoethyl phosphoramidite[2] (1.95 g, 5.32 mmol) was dissolved in anhydrous THF (12 mL) and activated with *1H*-tetrazole (373 mg, 5.32 mmol) for 10 minutes. The resulting mixture was then added dropwise to the tyrosine solution. The reaction was stirred overnight at room temperature in the dark. The mixture was filtered over celite and concentrated under reduced pressure, then re-dissolved in CH_2Cl_2 (75 mL), washed with 1% aqueous $NaHCO_3$ (2 x 40 mL), dried over Na_2SO_4, decanted and concentrated. The phosphite resdiue was then dissolved in anhydrous CH_2Cl_2 (20 mL) and *tBu*OOH (800 μL, 6M solution in decane) was added dropwise. After 35 minutes, the reaction mixture was diluted with 30 mL CH_2Cl_2, washed with 1% aqueous $NaHCO_3$ (2 x 30 mL), then dried with Na_2SO_4, decanted and concentrated. The resulting pale yellow oil was purified by silica gel flash chromatography (R_f = 0.50, 2:3 hex/EtOAc) to give the product as a clear oil (1.64 g, 77.0 %). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 1.32 (s, 9H), 1.64 (dd, 3H, J_{HH} = 6.3 Hz, J_{HH} = 13.6 Hz), 2.16-2.26 (m, 4H), 2.54-2.59 (m, 2H), 2.90-3.00 (m, 2H), 4.11-4.20 (m, 2H), 4.51-4.66 (m, 1H), 4.86-4.95 (m, 2H), 5.20-5.40 (m, 1H), 6.2-6.3 (m, 1H), 6.5-6.65 (m, 1H), 6.90-7.07 (m, 4H),

7.36-7.45 (m, 1H), 7.55-7.73 (m, 2H), 7.89 (d, 1H, J_{HH} = 8.2 Hz). ^{13}C-NMR (100.6 MHz, $CDCl_3$, δ): 171.9, 170.5, 148.8, 146.6, 136.8, 133.9, 130.8, 128.9, 127.5, 124.4, 119.6, 119.5, 115.4, 82.2, 77.4, 73.6, 62.5, 53.3, 37.1, 35.2, 29.3, 27.8, 24.1, 24.0, 19.4. ESI-MS: $[M+H]^+$ 602.1304 (obsd), 602.2267 (calcd).

N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl)-L-Tyrosine *tert*-butyl ester: N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-Tyrosine *tert*-butyl ester (400 mg, 0.66 mmol) was dissolved in methanol (MeOH, 15 mL) and potassium hydroxide (KOH, 1.3 mL of stock solution 34 mg/mL in water, 0.79 mmol) was added. After 10 minutes, TLC (R_f = 0.30, 1:10:90 Et_3N/MeOH/CH_2Cl_2) shows that the starting material has been consumed. The reaction mixture was poured into water (150 mL) with brine (50 mL) and acidified with HCl to pH ~ 1. The product was extracted with EtOAc (2 x 50 mL). The combined organic layers were dried over Na_2SO_4, decanted, and concentrated under reduced pressure to give the desired product as a white solid in quantitative yield. No further purification was necessary. ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 1.38 (s, 9H), 1.63 (dd, 3H, J_{HH} = 2.7 Hz, J_{HH} = 3.5 Hz), 2.24-2.32 (m, 4H), 2.95-3.03 (m, 2H), 4.68 (br s, 1H), 4.96 (dd, J_{HH} = 1.4 Hz, J_2 = 10.2 Hz),), 4.99 (dd, J_{HH} = 1.5 Hz, J_{HH} = 17.1 Hz), 5.70-5.78 (m, 1H), 6.08 (q, 1H, J_{HH} = 6.3 Hz), 6.27 (br s, 1H), 6.94-7.01 (m, 4H), 7.40 (t, 1H, J_{HH} = 8.1 Hz), 7.59 (t, 1H, J_{HH} = 7.5 Hz), 7.74 (dd, 1H, J_{HH} = 1.0 Hz, J_{HH} = 7.9 Hz), 7.93 (td, 1H, J_{HH} = 1.5 Hz, J_{HH} = 8.2 Hz), 10.85 (br s, 1H). ^{13}C-NMR (100.6 MHz, $CDCl_3$, δ): 172.5, 170.7, 149.6, 146.6, 137.9, 136.9, 134.1, 133.1, 130.8, 128.7, 128.0, 124.5, 120.0, 115.9, 82.8, 73.1, 53.7, 37.5, 35.5, 29.5, 28.1, 24.5. ESI-MS: $[M+H]^+$ 549.1981 (obsd), 549.1996 (calcd).

N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl)-L-Tyrosine cyanomethyl ester (6): N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl)-L-Tyrosine *tert*-butyl ester (400 mg, 0.81 mmol) was dissolved in anhydrous CH_2Cl_2 (10 mL) and the resulting solution was cooled to 0 °C. TFA (10 mL) was slowly added to the reaction mixture, and the solution was stirred in the dark at 0 °C for 30 minutes, and then at room temperature for another 30 minutes. The reaction mixture was concentrated under reduced pressure, then re-dissolved in CH_2Cl_2 and concentrated three times to remove the TFA. The resulting residue was dissolved in chloroacetonitrile (1 mL, 15.8 mmol), and DBU (607 μL, 4.06 mmol) was added dropwise over the mixture; the reaction was allowed to stir in the dark overnight. The reaction mixture was then diluted with EtOAc (50 mL) and washed with a mixture of water (50 mL), brine (150 mL) and 6M HCl (2 mL). The combined organic layers were dried over Na_2SO_4, decanted, and concentrated under reduced pressure to give a dark brown oil that was purified by reversed phase HPLC to give the desired product as a sticky solid in 47.0 % yield (201 mg, 95:5 to 5:95 water/acetonitrile/0.1%TFA over 30 minutes, R_t = 22.50 min). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 1.65 (dd, 3H, J_{HH} = 5.2 Hz, J_{HH} = 0.86 Hz), 2.29 (s, 4H), 2.96, 3.12 (m, 2H), 4.97 (d, 2H, J_{HH} = 1.1 Hz), 4.98 (dd, 2H, J_{HH} = 26 Hz, J_{HH} = 1.0 Hz), 5.64-5.79 (m, 1H), 6.06 (q, 1H, J_{HH} = 6.6), 6.72 (br s, 1H), 6.96-6.99 (m, 4H), 7.42 (td, 1H, J_{HH} = 7.7 Hz, J_{HH} = 1.3 Hz), 7.61 (td, 1H, 7.6 Hz, J_{HH} = 0.95 Hz), 7.76 (dd, 1H, J_{HH} = 7.8, J_{HH} = 0.97), 7.92 (d, 1H, J_{HH} = 8.2 Hz), 10.4 (br s, 1H). ^{13}C-NMR (100.6 MHz, $CDCl_3$, δ): 174.1, 170.1, 150.0, 146.7, 137.8, 136.4, 134.2, 132.1, 130.6, 128.9, 128.1, 124.5, 120.5, 116.2, 114.1, 73.0, 53.4, 49.2, 36.7, 35.1, 29.4, 24.5. ESI-MS: $[M+H]^+$ 532.1477 (obsd), 532.1485 (calcd).

Coupling to pdCpA (11): pdCpA (10) was synthesized as previously reported.[3] In general, the coupling reactions were performed on a 20 to 50 μmole scale; additionally, the pdCpA (1.2 equivalents), N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl)-L-Serine, -Threonine, or -Tyrosine cyanomethyl ester (1.0 equivalents), and tetrabutylammonium acetate (NBu_4OAc) were dried under vacuum before use. A 250 mM solution of NBu_4OAc was made in anhydrous *N,N*-dimethylformamide (DMF). The pdCpA was dissolved to 130 mM concentration with the NBu_4OAc solution, and kept under inert atmosphere. This mixture was then added to the amino acid ester, and kept dark under inert atmosphere. The reaction was monitored by analytical reversed phase HPLC. When the amino acid ester was consumed, the reaction was quenched with 2:1 water/MeCN with 0.1 % TFA (1.5 to 2.0 mL, or ~12 mM final concentration of product). The product was purified by reversed phase HPLC (93:7 to 0:100 water/MeCN/0.1 % TFA over 35 minutes) and confirmed by ESI-TOF MS. pdCpA-4PO-cpSer, R_t = 23.23 min, $[MNa]^+$ 1059.0, $[MNaH]^{2+}$ 530.0 (obsd), 1057.7, 529.3 (calcd); pdCpA-4PO-cpThr, R_t = 23.40 min, $[MNa]^+$ 1072.9, $[MH]^+$ 1049.0 (obsd), 1072.7, 1049.7 (calcd). pdCpA-4PO-cpTyr, R_t = 25.80 min, $[MH]^+$ 1111.2, $[M+2H]^{2+}$ 556.1 (obsd), 1111.0, 556.0 (calcd).

Biochemical Synthesis and Molecular Biology

Ligation to $tRNA_{CUA}^{-CA}$: $tRNA_{CUA}^{-CA}$ was transcribed from linearized DNA as previously reported[4] and stored in diethyl pyrocarbonate (DEPC) treated water at – 80 °C. Ligations were performed as published[1, 4, 5] with two changes: the ligation procedure was increased 1.5-fold, and the following desalting step was added. After redissolution in 20 μL 1mM NaOAc, pH 4.5, aminoacyl-$tRNA_{CUA}$ samples were desalted using BD Biosciences CHROMA SPIN-30 DEPC H2O columns (following manufacturer's instructions). The desalted samples were quantified using UV A_{260}, confirmed by MALDI-TOF MS on 3-hyroxypicolinic acid (3-HPA) matrix[1], and stored at – 80 °C.

$tRNA_{CUA}$CpSer: $[MH]^+$ 24, 732 (obsd), 24, 734 (calcd).
$tRNA_{CUA}$CpThr: $[MH]^+$ 24, 755 (obsd), 24, 748 (calcd).
$tRNA_{CUA}$CpTyr: $[MH]^+$ 24, 807 (obsd), 24, 810 (calcd).
Calculated masses based on external BSA calibration, and internal referencing to 74 base $tRNA_{CUA}^{-CA}$ (23, 716) and 76 base $tRNA_{CUA}$ (24, 334). Other tRNAs produced as previously described.[5]

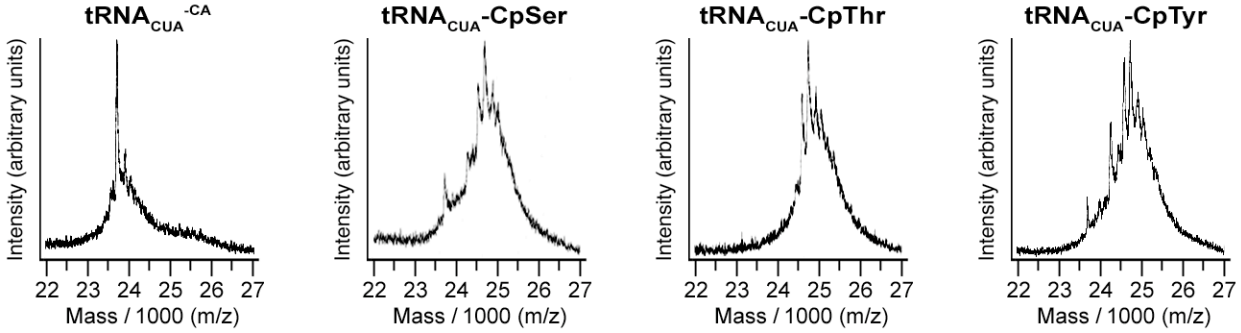


Figure S1 MALDI-TOF MS of $tRNA_{CUA}$.

VASP-WT and –S153TAG constructs: The VASP gene was received from the Gertler Lab (Massachusetts Institute of Technology) in a pBS-KSII vector with an upstream T5 promoter. The site-specific mutagenesis was performed following the Stratagene QuikChange protocol and the following primers: sense 5'-GAG CGC CGG GTC TAG AAT GCA GGA GGC CC; antisense 5'-GGG CCT CCT GCA TTC TAG ACC CGG CGC TC. Several colonies were selected, DNA was purified by standard procedure, and submitted for sequencing; one sample with the correct sequence was kept for further use.

Both the WT and S153TAG VASP genes were amplified from the pBS-KSII vectors using PCR with *Pfu* Turbo Polymerase and the following primers: forward 5'-C ACC ATG ACG GAG ACG GTC; reverse 5'-AGG AGA ACC CCG CTT CCT CAG. The products were purified by agarose gel electrophoresis and stored at -20 °C. The PCR products were then used in the TOPO directional cloning reaction as described by the manufacturer (Invitrogen, TOPO Directional Cloning: pcDNA3.1/V5-His). Several colonies were selected and plasmid DNA purified by standard procedure. The DNA was screened by a single restriction enzyme digest: Sal I will cut the empty vector into two pieces, 2.3 and 3.2 kb in length, while it will cut the pcDNA/VASP vectors into two pieces, 2.1 and 4.4 kb in length. Several samples with the insert were submitted for sequencing, and one of each plasmid sample with the correct sequence was stored at –20 °C for future use.

mRNA run-off transcription: Plasmid DNA of WT and S153TAG VASP was amplified in DH5α cells using standard protocol. Approximately 100 μg of each plasmid were linearized with PmeI (cuts immediately downstream of the C-terminal His-tag). Linearized DNA was purified by agarose gel electrophoresis followed by PCI extraction, ethanol preceipitation, and re-dissolving in DEPC water. Run-off transcription of the genes was performed using the T7 mMessage mMachine Kit (Ambion) and following manufacturer protocol. The mRNA was purified by PCI extraction and stored at –80 °C. Analysis by gloxal/agaorse gel confirmed the correct size of the transcripts. nAChR transcripts were run off as previously described.[6]

Aminoacyl-$tRNA_{CUA}$ Deprotection: 6-nitroveratryloxycarbonyl- (NVOC) protected aminoacyl-$tRNA_{CUA}$s (1 μg/μL) were deprotected by 5 minutes of irradiation with a 1000 W Hg/Xe arc lamp (Oriel, Stratford, CT) operating

at 400 W equipped with WG-335 and UG-11 filters (Schott, Elmsford, NY). 4-pentenoyl- (4PO) protected aminoacyl-$tRNA_{CUA}$s were deprotected by a 10 minute incubation with 1 equivalent fresh saturated I_2 in H_2O. 4PO-protected aminoacyl-$tRNA_{CUA}$s are stored at 2 μg/μL; the final concentration is 1 μg/μL after I_2 treatment.

***In Vitro* Translation:** *In vitro* protein synthesis was carried out using the Promega (Madison, WI) rabbit reticulocyte lysate translation kit according to the manufacturer's instructions. For nAChR translations, the following reagents were combined and incubated for 1 hour at 30 °C: 1.5 μL DEPC H_2O, 0.75 μL complete amino acids (1mM), 0.5 μL Roche (Basel, Switzerland) RNAse inhibitor, 4 μL deprotected aminoacyl-$tRNA_{CUA}$ (suppression: 1 μg/μL) or DEPC H_2O (unsuppressed), 1 μL mRNA (VASP-WT: 1.0 μg/μL, VASP-S153TAG: 0.1 μg/μL), and 16.25 μL lysate. For VASP translations, the following reagents were combined and incubated for 3 hours at 30 °C: 10 μL DEPC H_2O, 6 μL complete amino acids (1mM), 4 μL Roche (Basel, Switzerland) RNAse inhibitor, 32 μL deprotected aminoacyl-$tRNA_{CUA}$ (suppression: 1 μg/μL) or DEPC H_2O (unsuppressed), 8 μL mRNA (VASP-WT: 1.0 μg/μL, VASP-S153TAG: 0.1 μg/μL), and 140 μL lysate.

Ni-NTA Purification of His-Tagged Proteins: The following buffers were used in the purification and concentration of His-tagged VASP proteins:

- Buffer A: 100 mM NaH_2PO_4, 2% SDS, 2 mM β-mercaptoethanol, pH 8.0
- Buffer B: 50 mM NaH_2PO_4, 150 mM NaCl, 2 mM β-mercaptoethanol, pH 8.0
- Buffer C: 50 mM NaH_2PO_4, 150 mM NaCl, 2 mM β-mercaptoethanol, pH 7.0
- Buffer D: 50 mM NaH_2PO_4, 150 mM NaCl, 2 mM β-mercaptoethanol, pH 4.5
- Buffer E: 50 mM NaH_2PO_4, 150 mM NaCl, 2 mM β-mercaptoethanol, 1X BSA, pH 7.0.

400 μL Qiagen Ni-NTA Superflow (Valencia, CA) were washed three times with 400 μL Buffer B. 200 μL translation mix was combined with 400 μL Buffer A, added to the beads, and rocked at room temperature for two hours. The beads were washed four times with 400 μL Buffer C. 400 μL Buffer D was added to beads, which were rocked for two hours at room temperature. Buffer D was removed from the beads, another 400 μL Buffer D was added, and the beads were rocked at room temperature for two hours. Buffer D elutions were combined.

Three Millipore (Billerica, MA) Ultrafree MC Biomax (30 kDa NMWL) centricon columns were used per translation for concentrating solutions, one for loading Buffer A, one for Buffer C washes, and one for Buffer D elutions. Columns were blocked for 2 hours at room temperature with Buffer E, which was removed by pipetting. 2 X 400 μL Buffer B were added to each column and removed by pipetting (removes excess BSA). Loading Buffer A, Buffer C washes, and Buffer D elutions were added to the columns and concentrated by centrifuging the columns at 4000 x *g* until their respective volumes were reduced to 40 μL. Buffer B was used to dilute the concentrated Buffer A, Buffer C, and Buffer D solutions to 100 μL (final pH ~6.6). Samples stored at –80 °C. Concentrated Buffer D solutions are hereafter referred to as VASP-WT (unsuppressed) or Suppressed VASP.

λ–PPase Treatment: λ-PPase dephosphorylation of murine VASP Ser153 is well-precedented.[7] Dephosphorylation was performed with New England Biolabs (Beverley, MA) Lambda Protein Phosphatase (λ-PPase), according to the manufacturer's instructions. VASP samples (WT: 2 μL, Suppressed: 10 μL) were dissolved in sterile H_2O to a total volume of 15.5 μL. 2 μL 20 mM $MnCl_2$, 2 μL λ-PPase Buffer, and 0.5 μL λ-PPase enzyme were added to a total volume of 20 μL. This mixture was incubated 0.5 hours at 30°C and stored at -80°C.

PKA treatment: PKA phosphorylation of murine VASP Ser153 is well-precedented.[7] Phosphorylation was performed with New England Biolabs cAMP-dependent Protein Kinase (PKA), according to the manufacturer's instructions. VASP samples (WT: 2 μL, Suppressed: 10 μL) were dissolved in sterile H_2O to a total volume of 15.5 μL. 2 μL 10 mM ATP, 2 μL PKA Buffer, and 0.5 μL PKA enzyme were added to a total volume of 20 μL. This mixture was incubated 0.5 hours at 30°C and stored at -80°C.

Caging Group Photolysis: Irradiation of the His-Tag purified VASP samples was performed with the arc lamp assembly described above (Aminoacyl-$tRNA_{CUA}$ Deprotection). WT samples were treated with λ-PPase as described above; after dephosphorylation the 20 μL sample was irradiated for 5 minutes at room temperature. CSer and CpSer samples were diluted with sterile H_2O to a total volume of 15.5 μL, irradiated for 5 minutes at room temperature, and then treated with PKA or λ-PPase (respectively) as described above.

nAChR PAGE Analysis: PAGE samples were prepared by mixing 10 μL of unpurified rabbit reticulocyte translation mix with 4 μL 6X SDS gel loading buffer[8] and diluting to a total volume of 24 μL. Samples were run at

150 V on a 10% polyacrylamide Readygel (BioRad, Hercules, CA) in 1X Tris/Glycine/SDS buffer (10X stock from BioRad). Protein was transferred to nitrocellulose membrane, which was blocked with 5% milk in 1 X PBS (Irvine Scientific, Santa Ana, CA) with 0.1 % (v/v) Tween 20 (Sigma-Aldrich) for 1 hour. The blot was labeled with mouse anti-HA epitope primary antibody (Covance Research Products, Grand Rapids, MI) for 1 hour at a 1:3000 dilution in the 5% milk solution. After washing 3 times 1 X PBS + 0.1 % (v/v) Tween 20, the membrane was labeled for 1 hour with horse radish peroxidase-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA). The blot was washed 3 times 1 X PBS + 0.1 % (v/v) Tween 20 and developed with Supersignal West Pico chemiluminescence reagents from Pierce (Rockford, IL) on Amersham (Buckinghamshire, England) Hyperfilm.

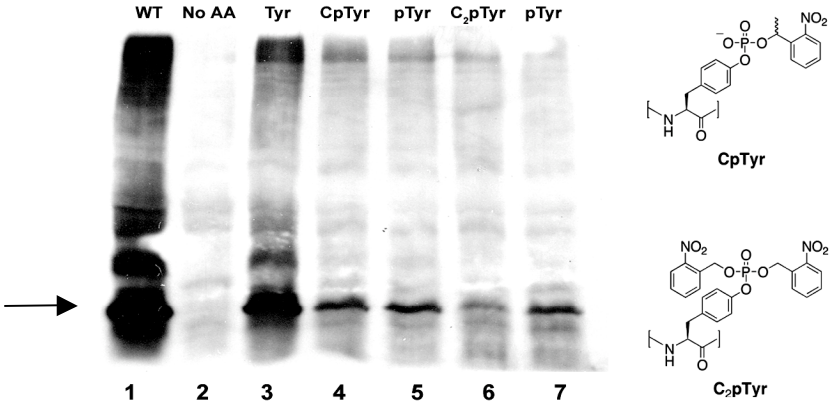


Figure S2 Suppression efficiency in nAChR-A122TAG. Lanes (Left to Right): 1, WT translation; 2-7, A122TAG mRNA with $tRNA_{CUA}$ charged with amino acids listed above lanes. "No AA" refers to full length, but uncharged, tRNA. All lanes loaded with 10 μL. C_2pTyr synthesis is described elsewhere.[9]

VASP PAGE Analysis: PAGE samples were prepared by mixing 4 μL 6X SDS gel loading buffer[8] with one of the following: 2 μL of unpurified rabbit reticulocyte translation mix; 10 μL concentrated loading Buffer A, wash Buffer C, or elution Buffer D; 20 μL enzymatically-treated VASP. The final samples were diluted to 24 µL. Samples were run at 150 V on a 12% polyacrylamide Readygel (BioRad) in Tris/Glycine/SDS. Protein was transferred to nitrocellulose membrane, which was blocked with 5% BSA (minimum 96%, Sigma-Aldrich, St. Louis, MO) in 1 X PBS with 0.1 % (v/v) Tween 20 for 1 hour. The blot was labeled with rabbit anti-VASP primary antibody (a gift from the Gertler lab, Massachusetts Institute of Technology) for 1 hour at a 1:3000 dilution in the 5% BSA solution. After washing 3 times 1 X PBS + 0.1 % (v/v) Tween 20, the membrane was labeled for 1 hour with horse radish peroxidase-conjugated goat anti-rabbit secondary antibody (Upstate, Charlottesville, VA). The blot was washed 3 times 1 X PBS + 0.1 % (v/v) Tween 20 and developed as above.

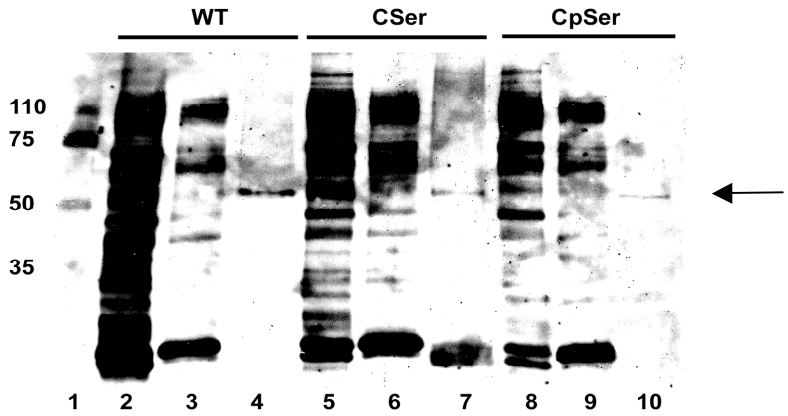
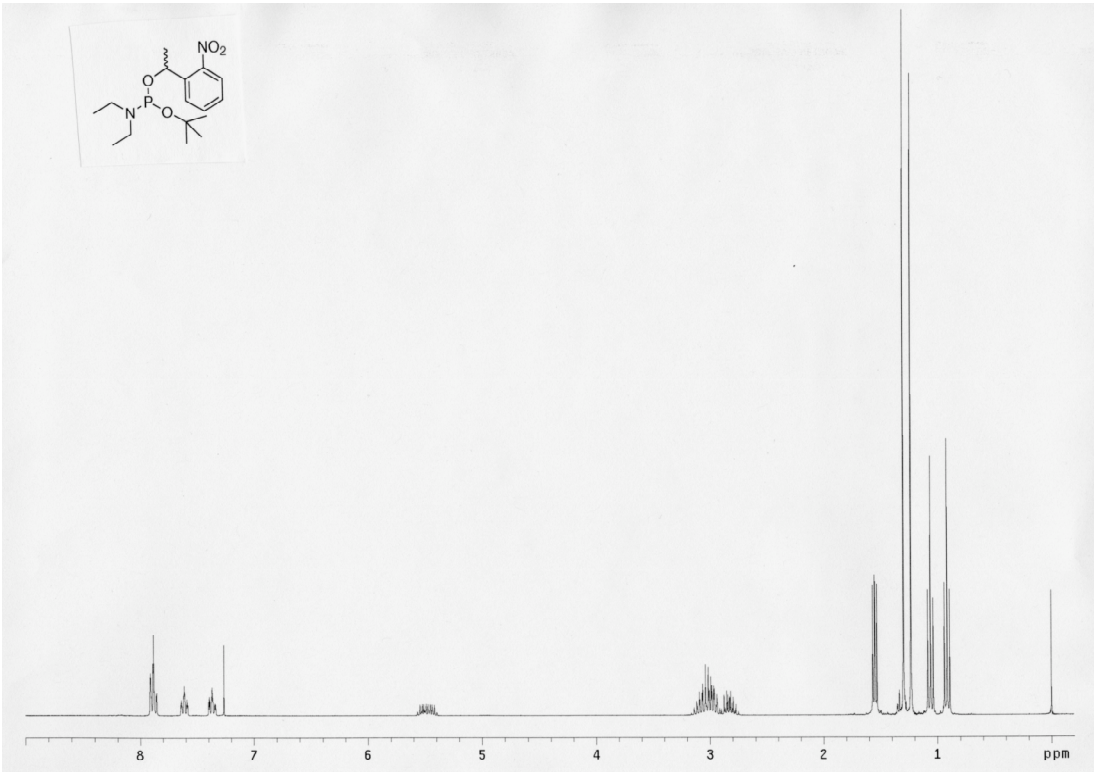
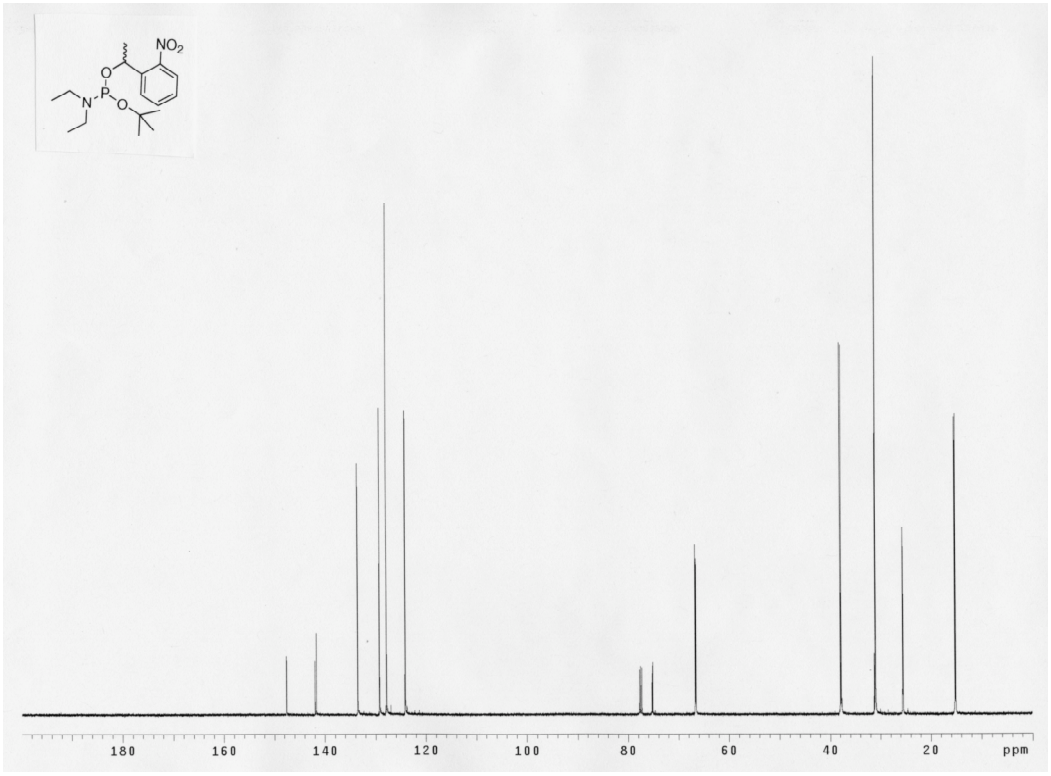


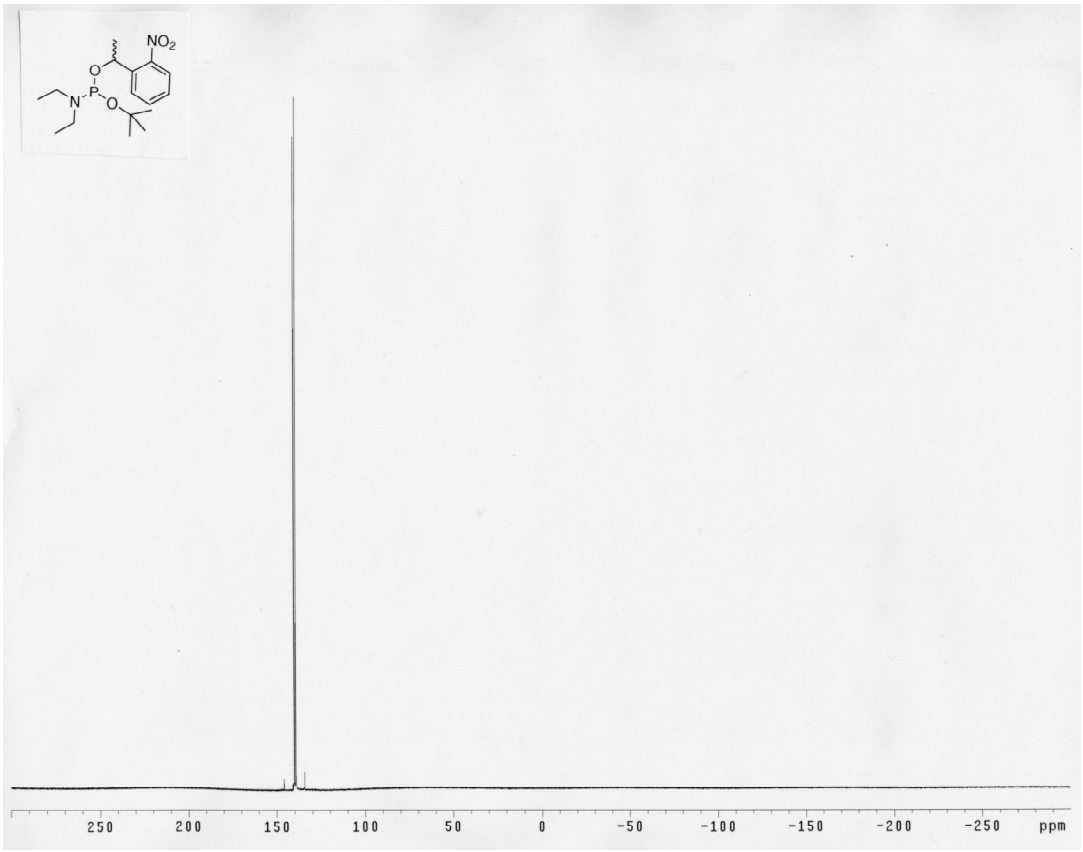
Figure S3 Suppression efficiency in VASP-S153TAG. Lanes (Left to Right): 1, Molecular Weight Markers; 2, WT translation mix, unpurified (2 μL); 3, WT Buffer C Wash (10 μL); 4, WT Buffer D elution (2 μL); 5, S153TAG mRNA and $tRNA_{CUA}$-CSer translation mix, unpurified (2 μL); 6, CSer Buffer C Wash (2 μL); 7, CSer Buffer D elution (10 μL); 8, S153TAG mRNA and $tRNA_{CUA}$-CpSer translation mix, unpurified (2 μL); 9, CpSer Buffer C Wash (2 μL); 10, CpSer Buffer D elution (10 μL).

NMR Data

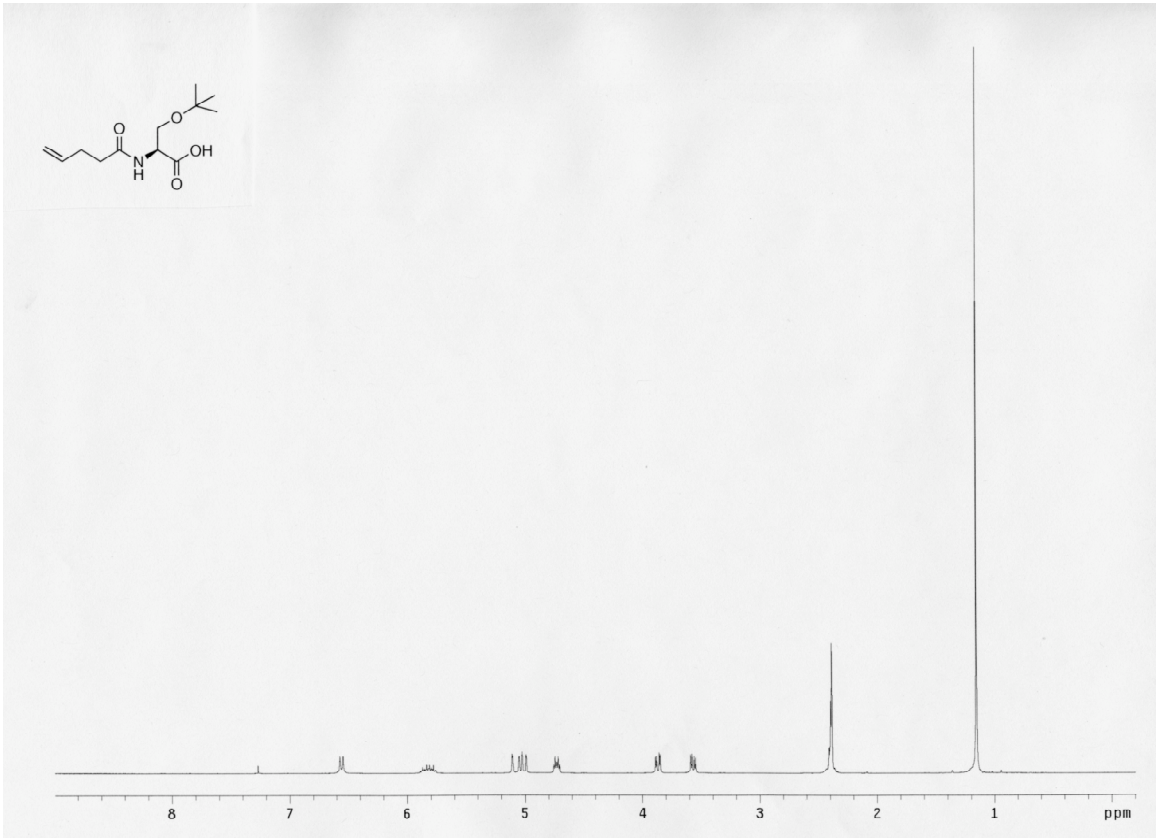
_O_-1-(2-nitrophenyl)ethyl-_O'_-tertbutyl-_N,N_-diethyl phosphoramidite (3)

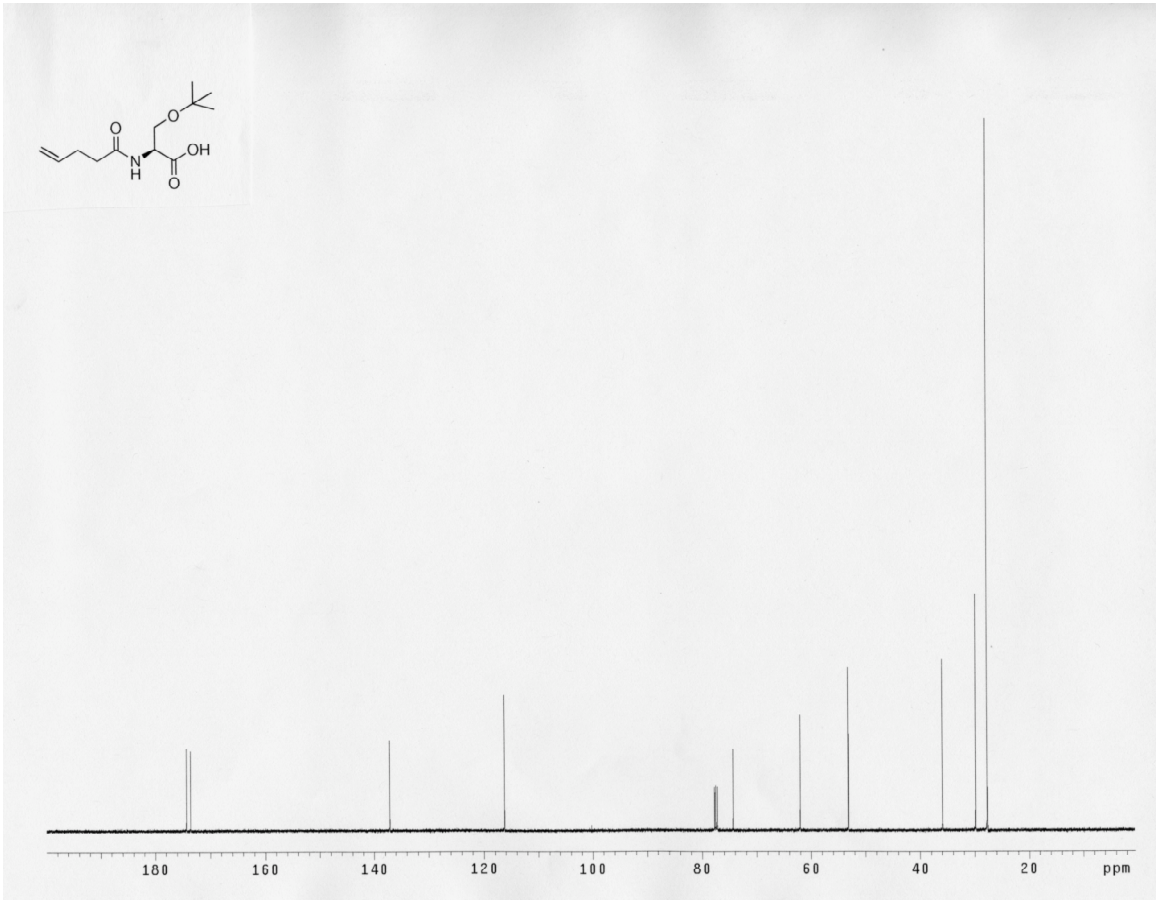




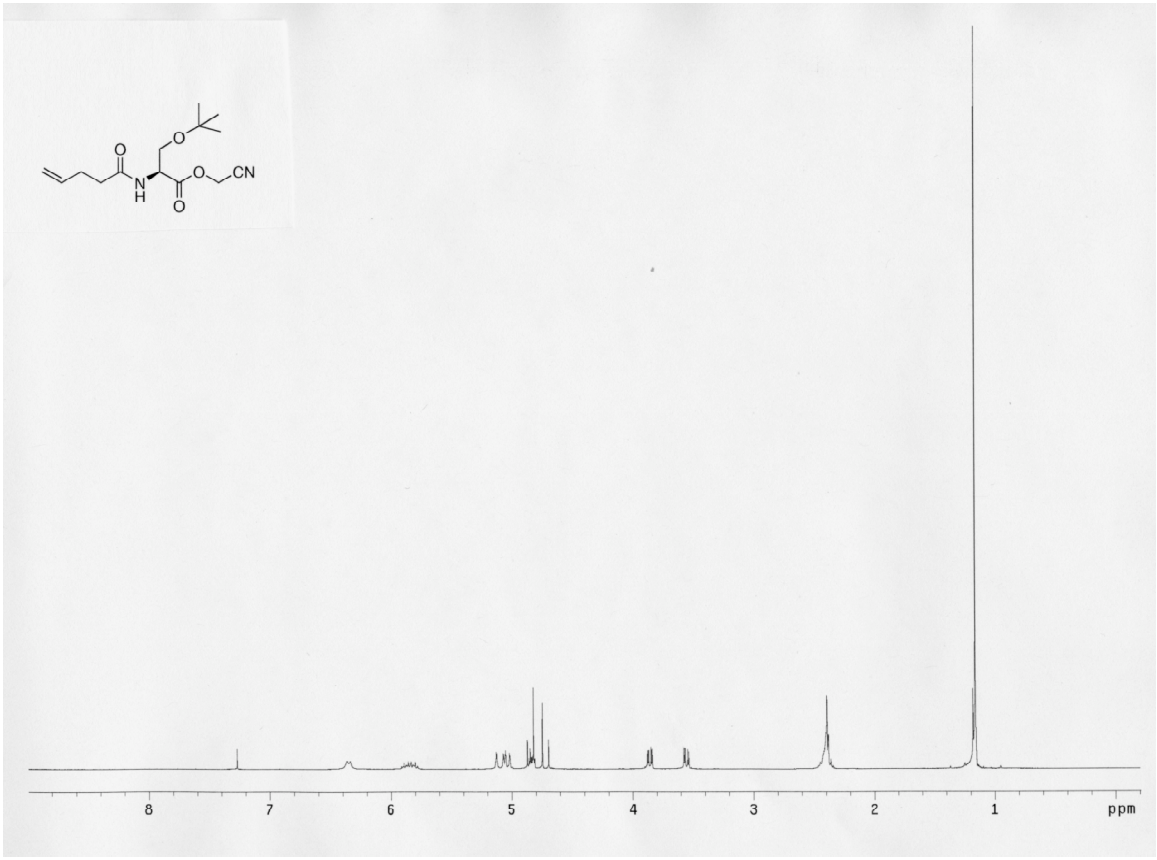


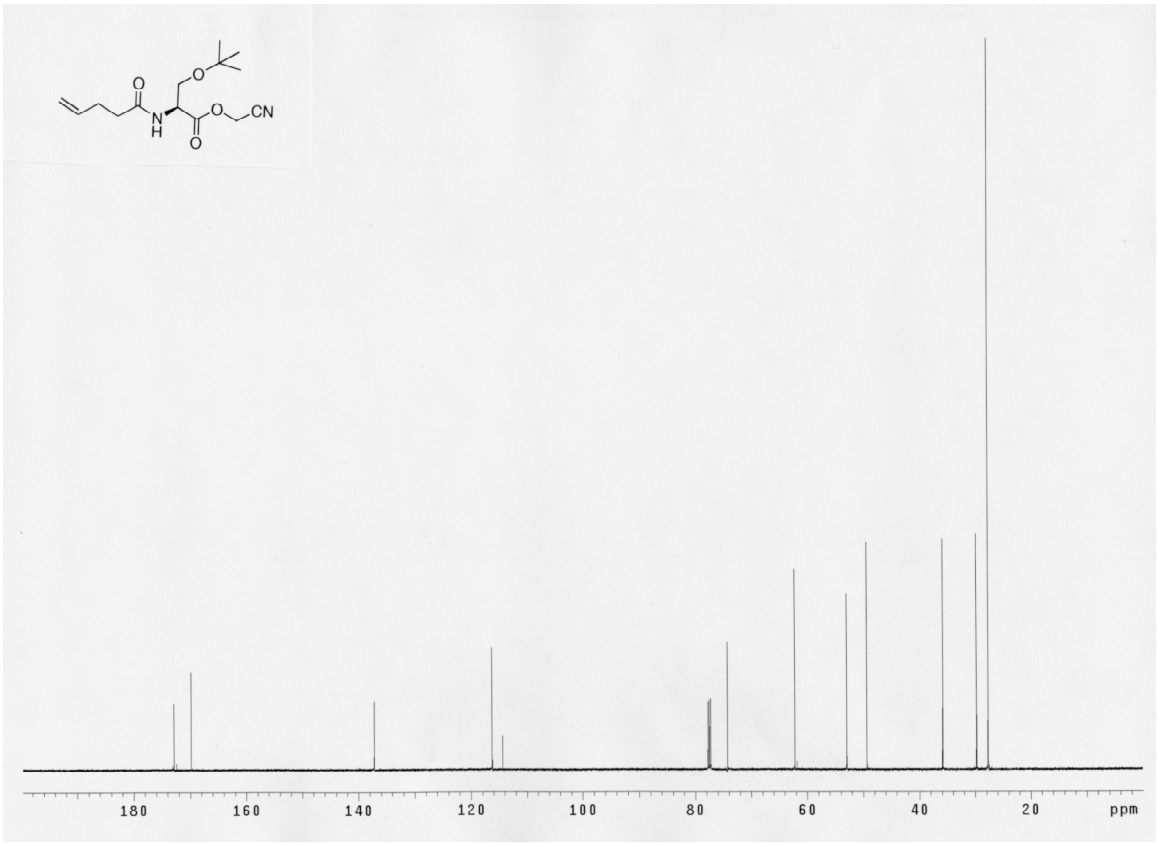
N^{α}-4-pentenoyl-*O-tert*-butyl-L-Serine



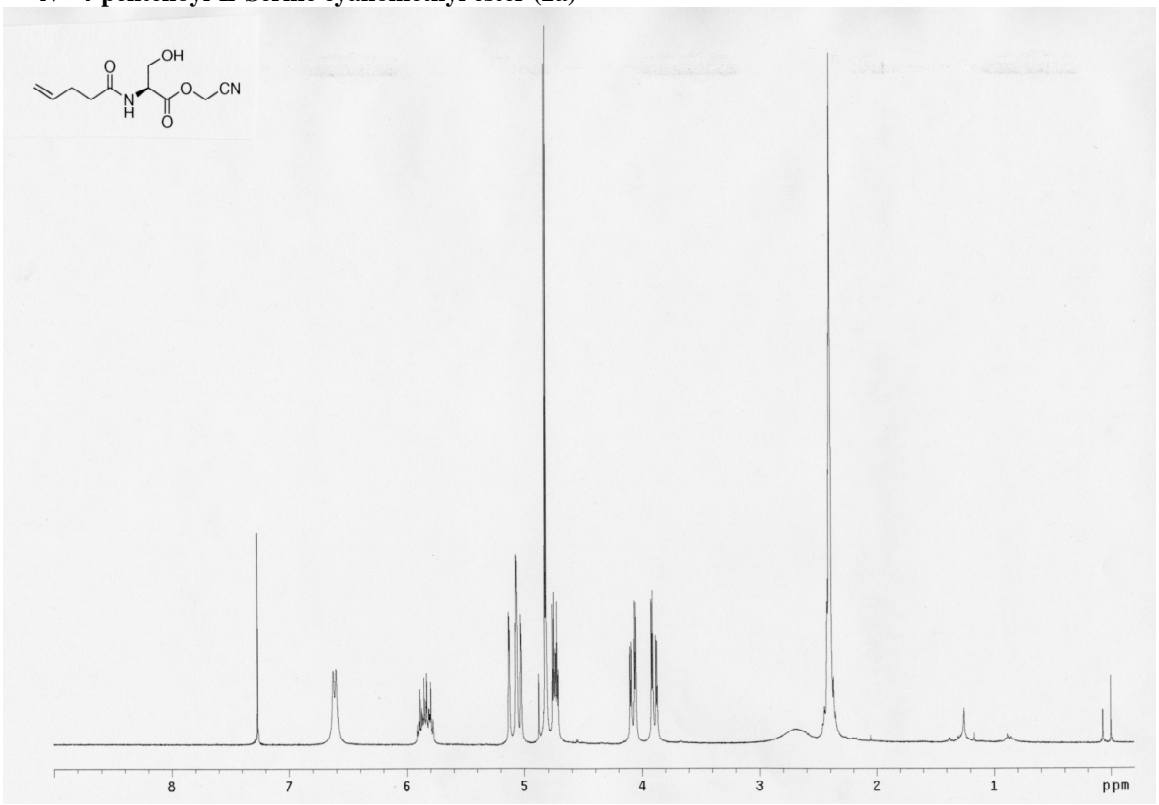


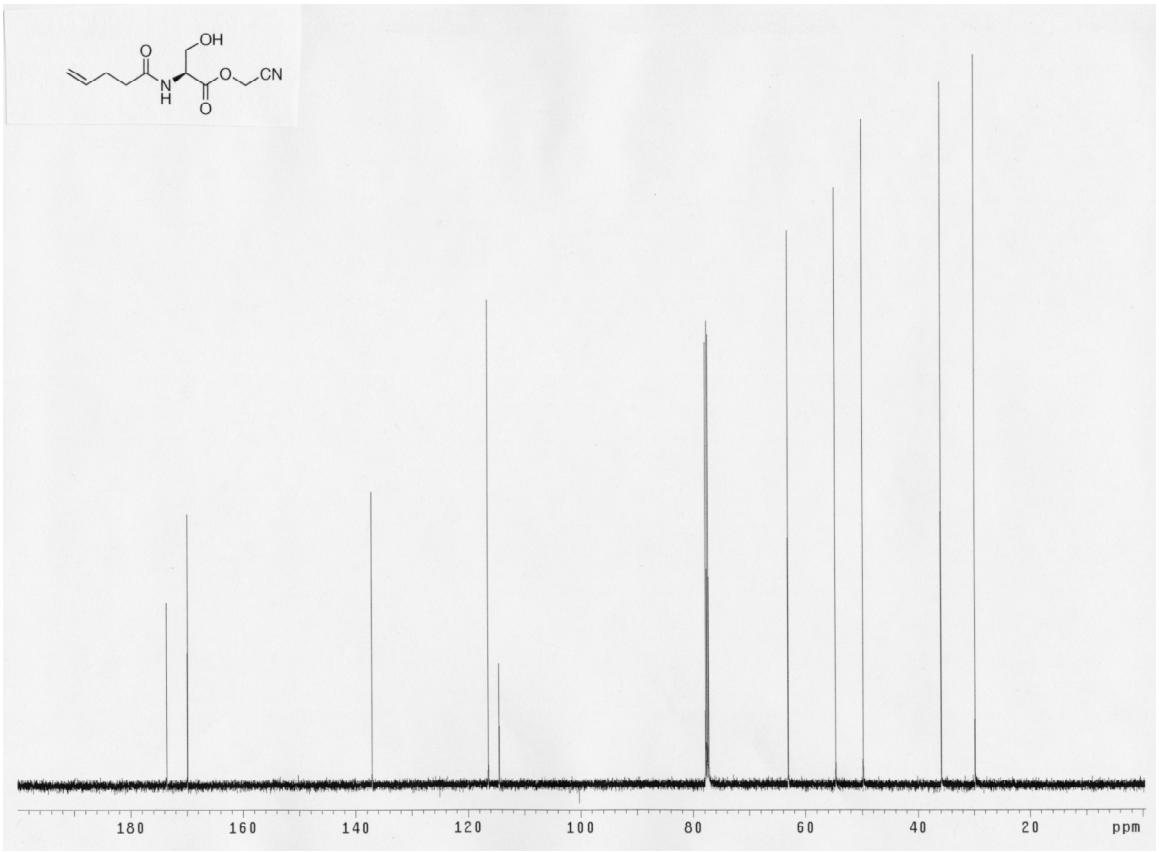
N^{α}-4-pentenoyl-*O-tert*-butyl-L-Serine cyanomethyl ester



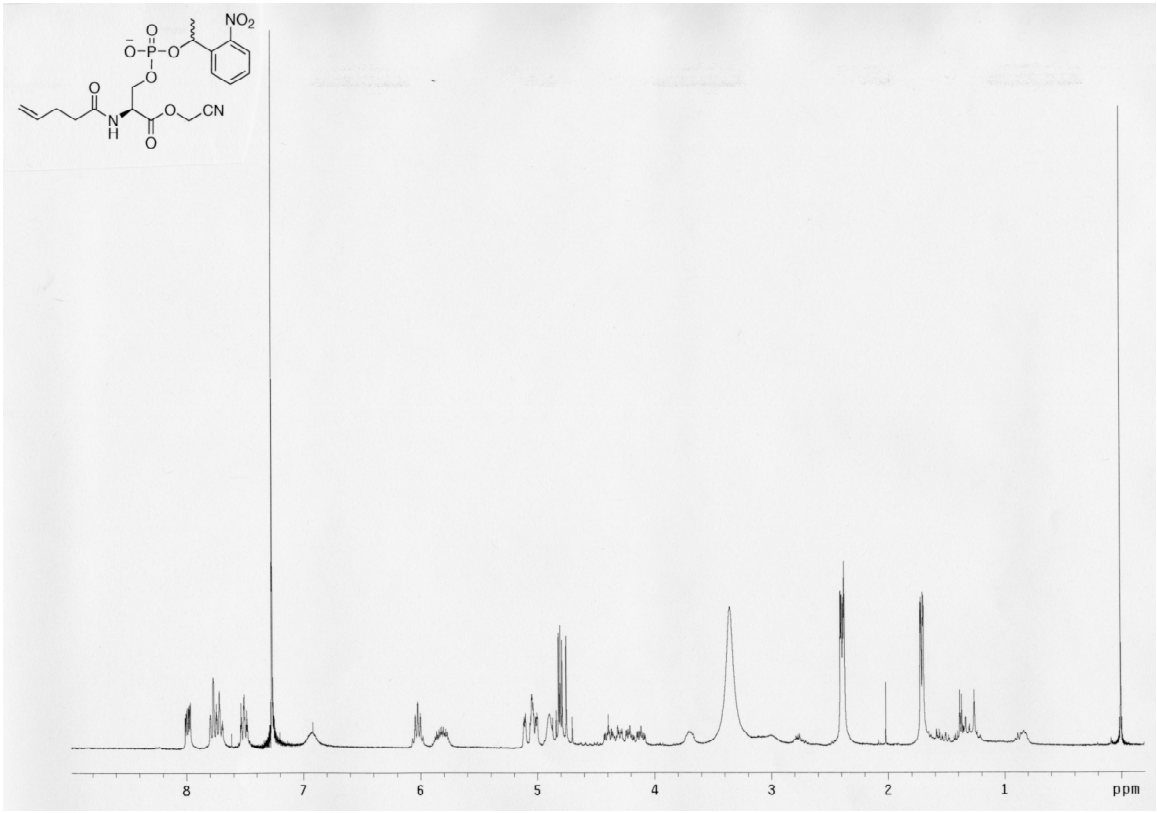


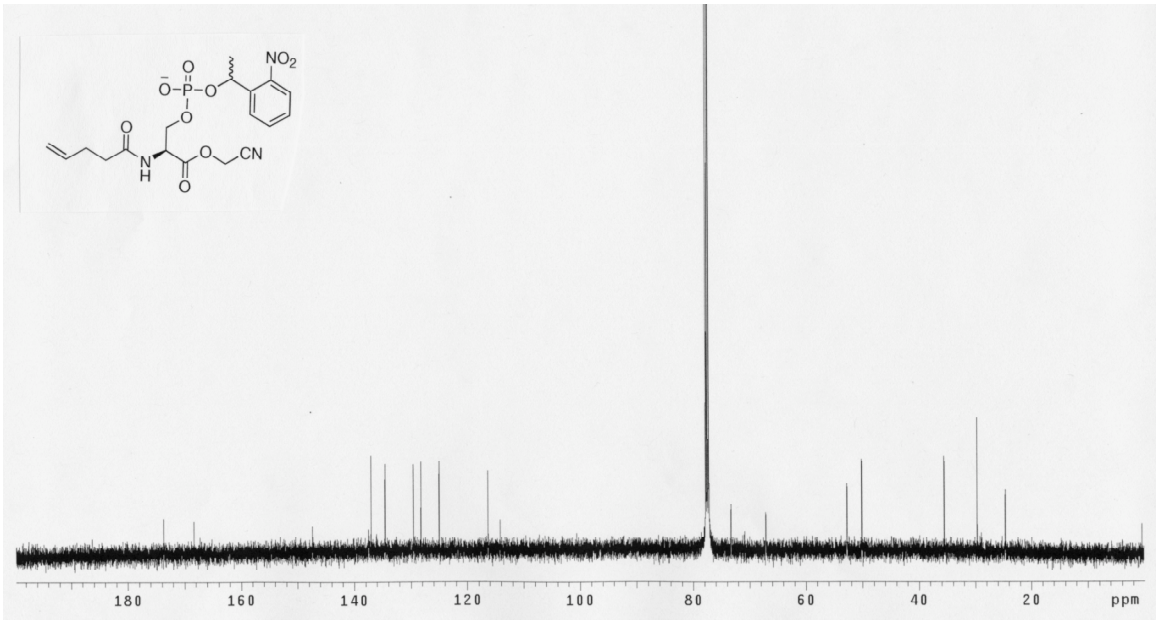
N^{α}-4-pentenoyl-L-Serine cyanomethyl ester (2a)





***N*$^{\alpha}$-4-pentenoyl-phospho(1-nitrophenylethyl)-L-Serine cyanomethyl ester (5a)**

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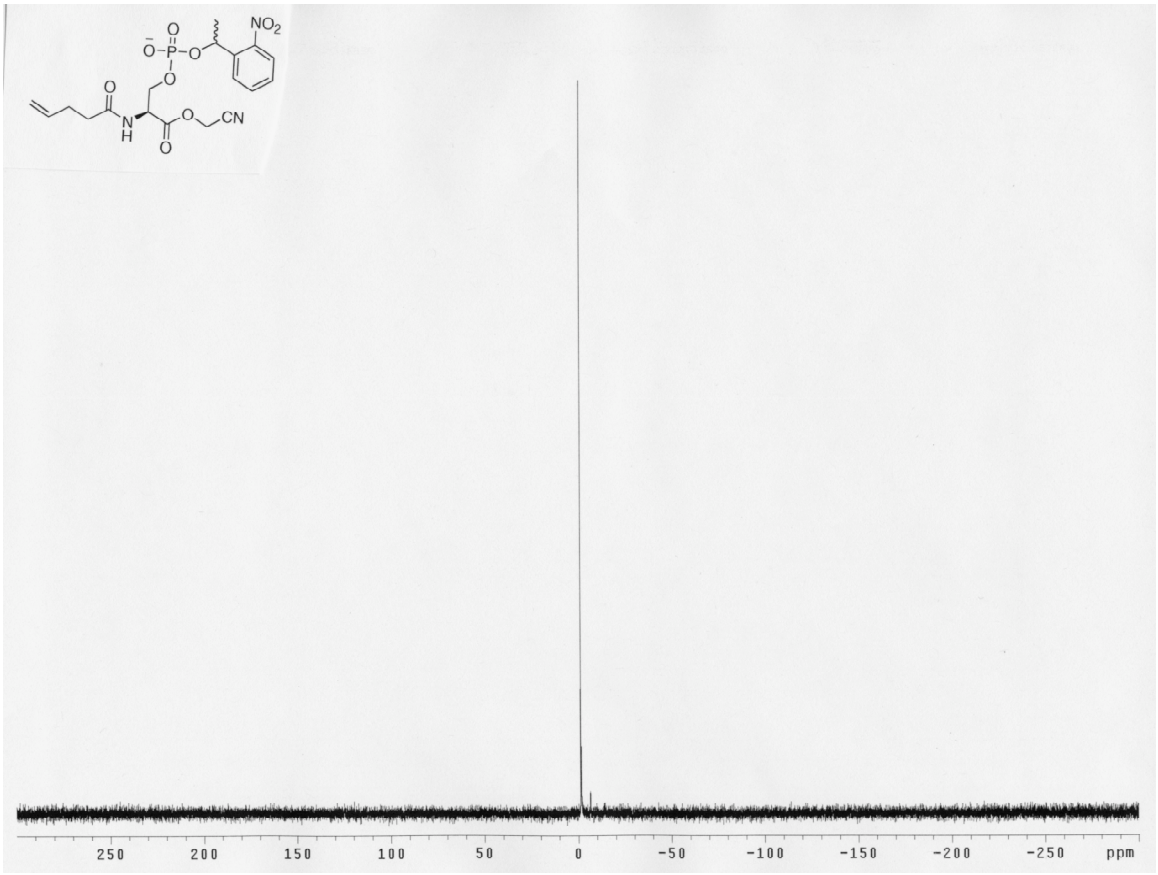
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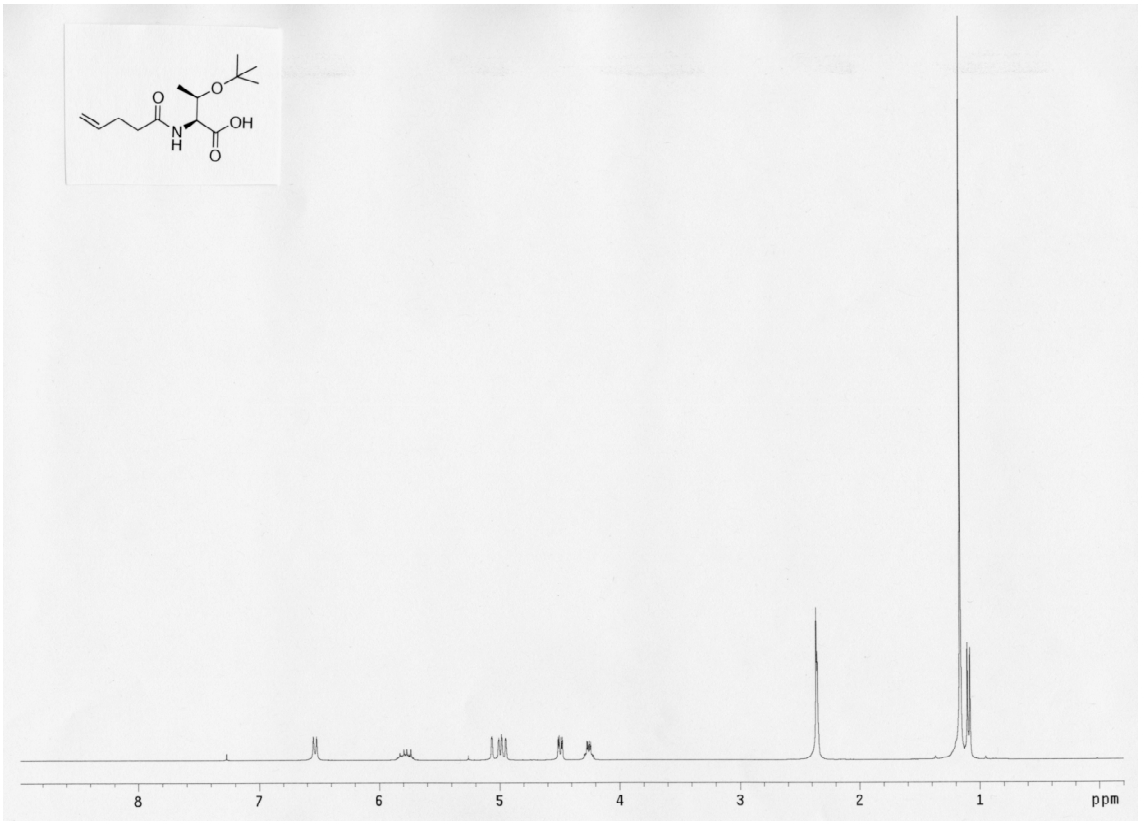
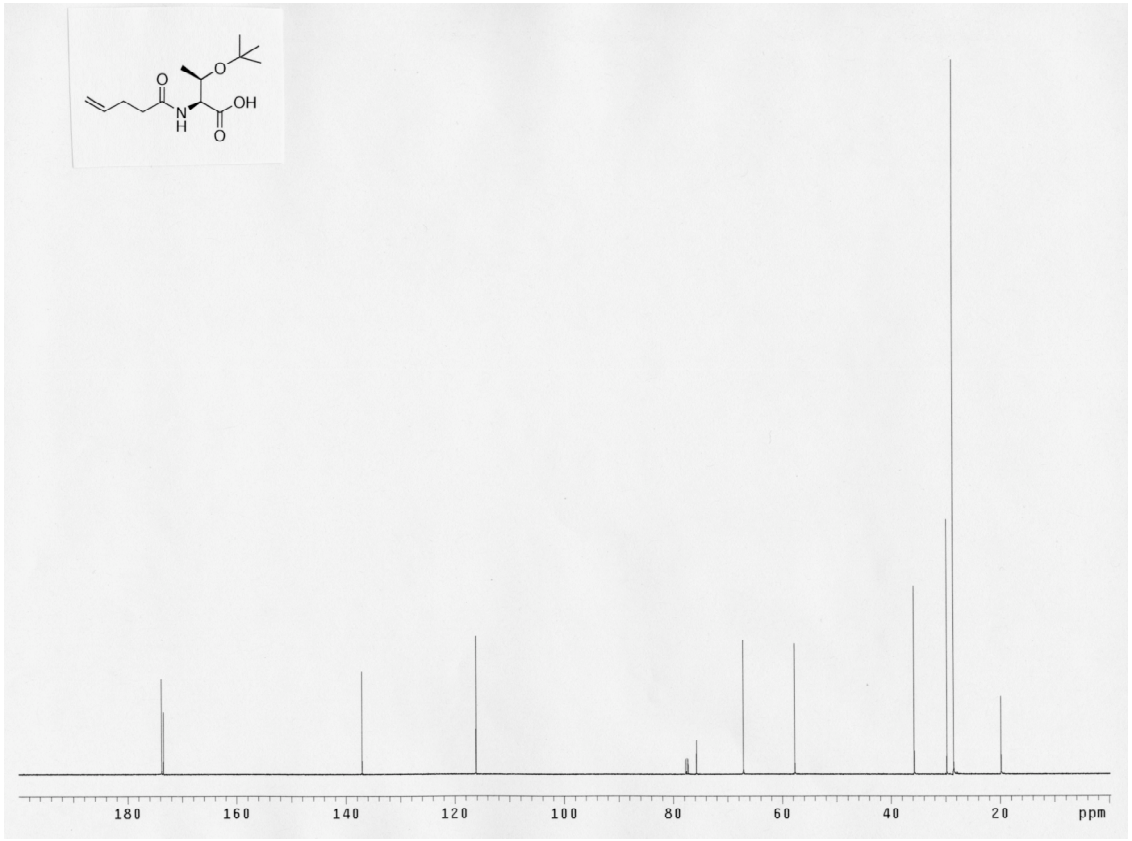
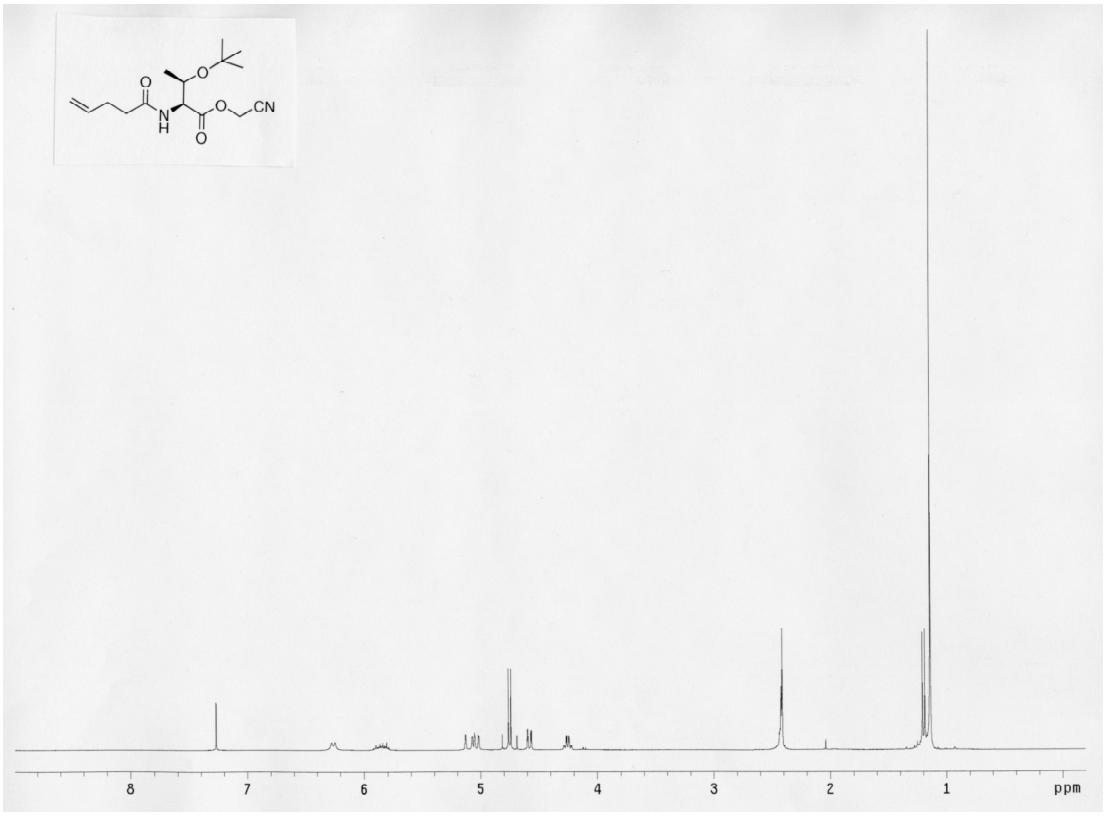
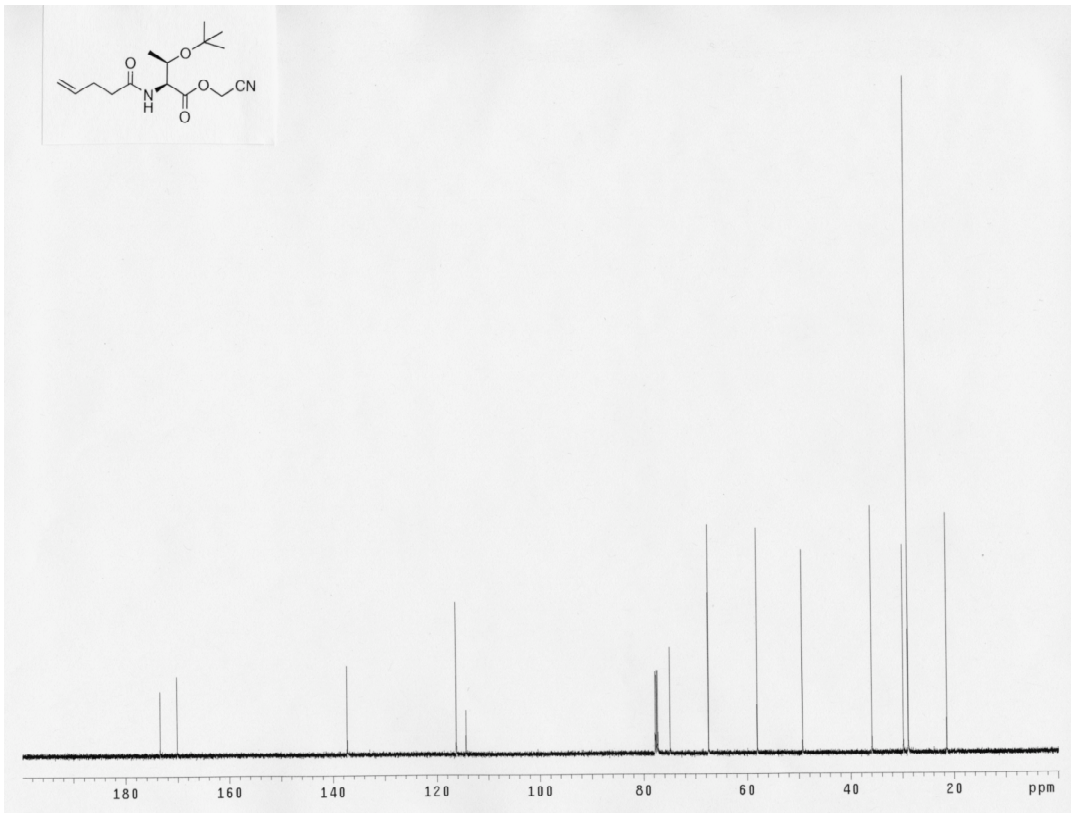
N^{α}-4-pentenoyl-*O-tert*-butyl-L-Threonine



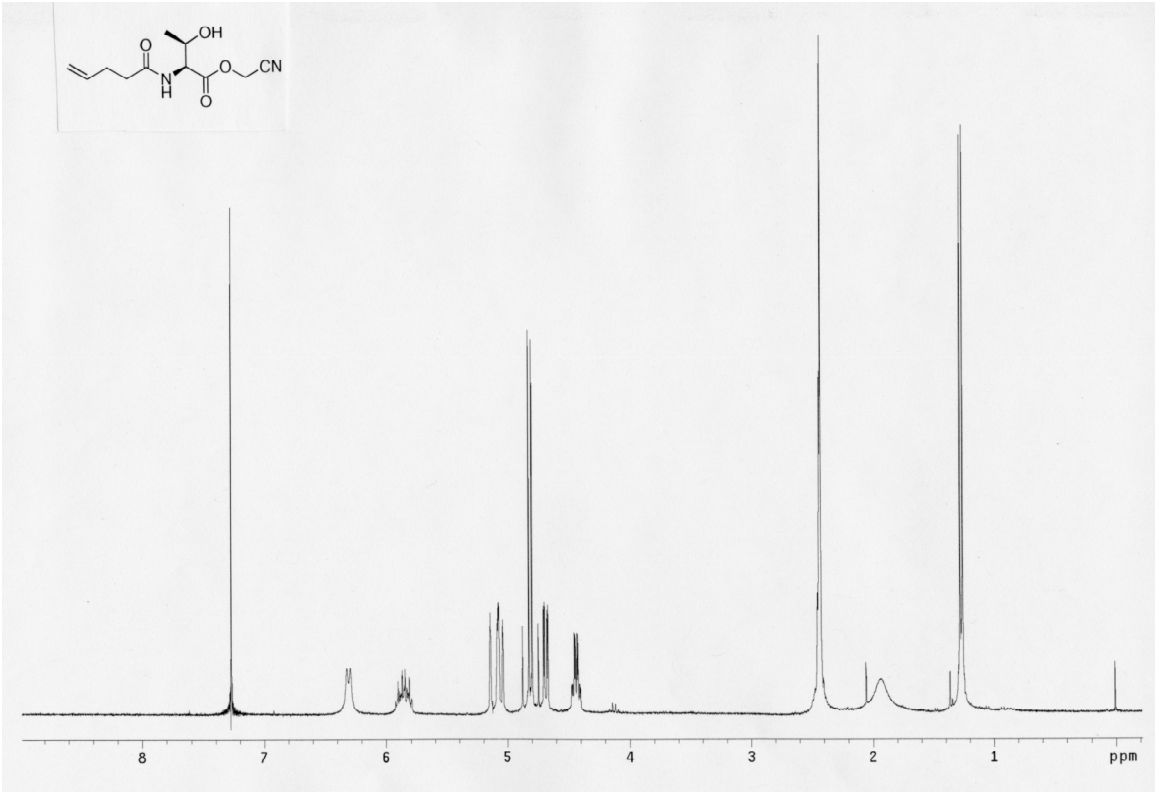


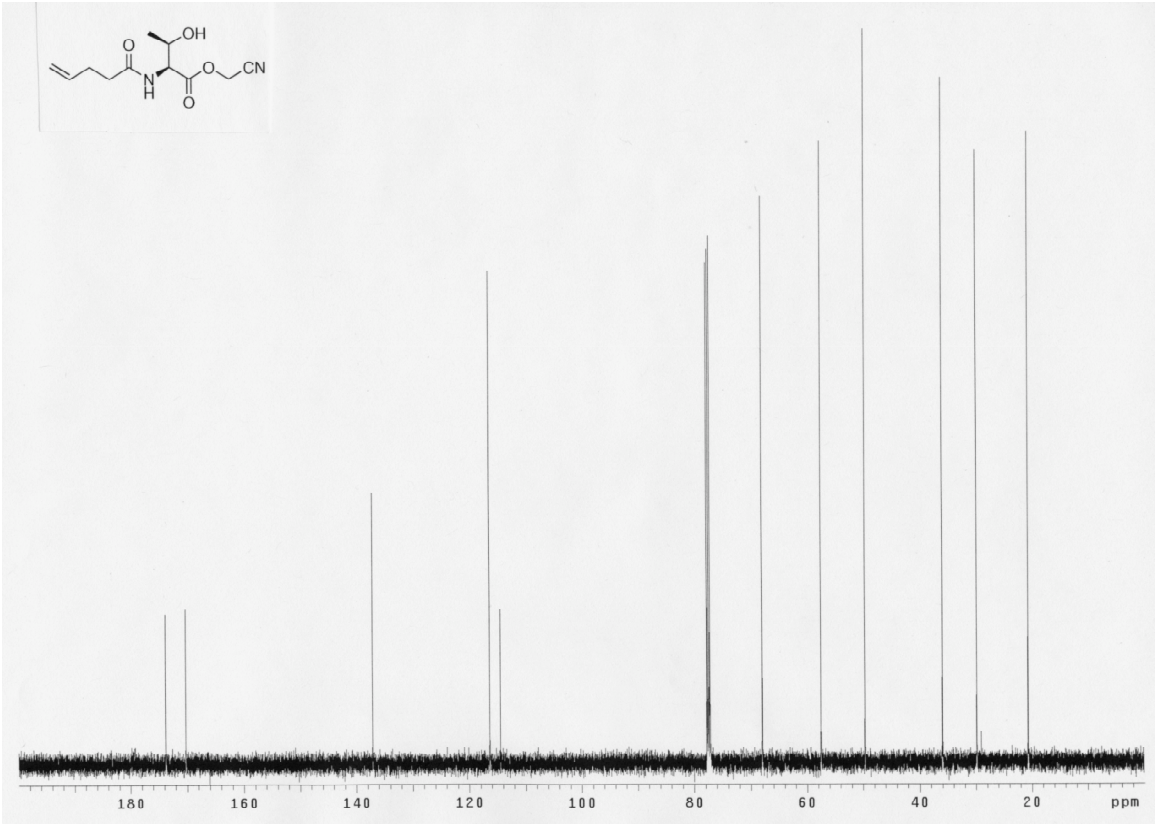
N^{α}-4-pentenoyl-*O-tert*-butyl-L-Threonine cyanomethyl ester



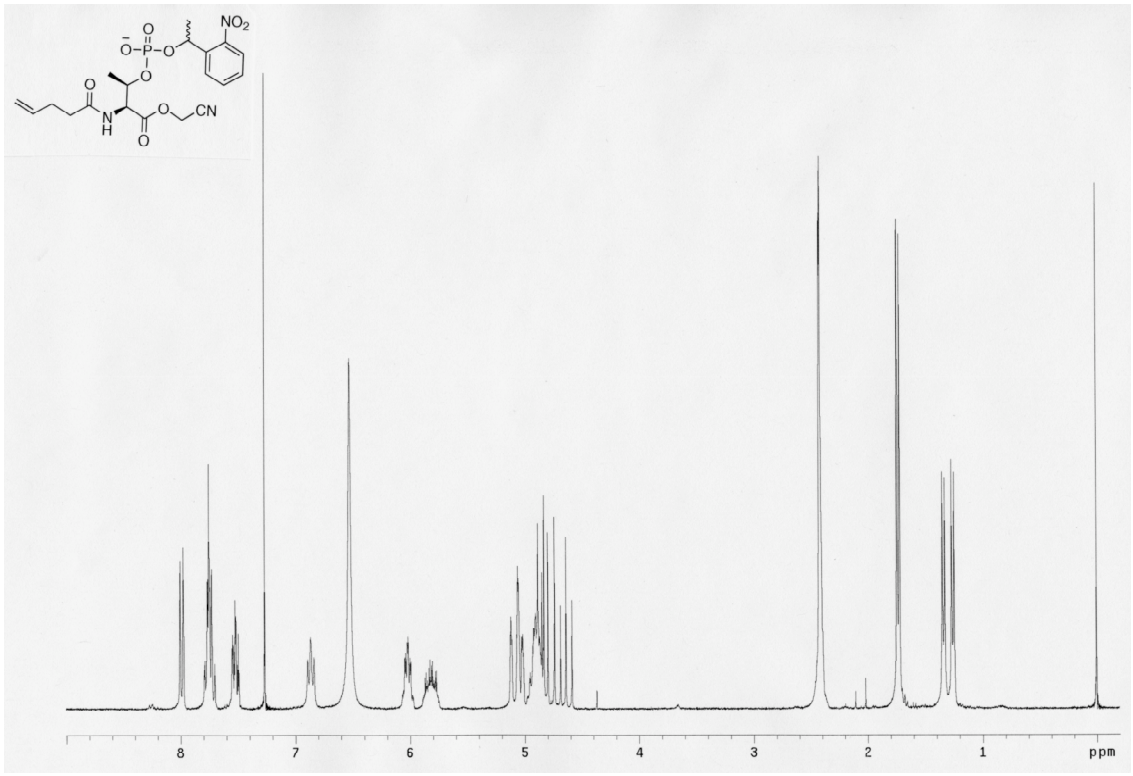


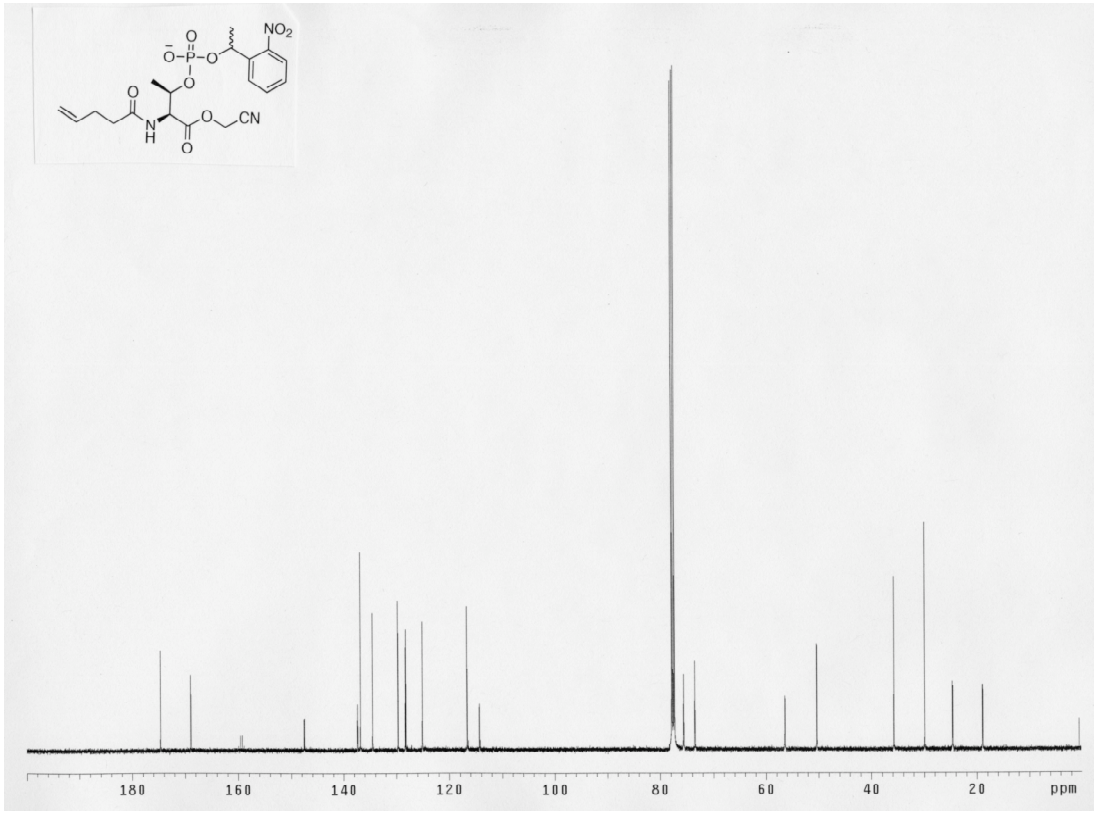
***N*$^{\alpha}$-4-pentenoyl-L-Threonine cyanomethyl ester (2b)**

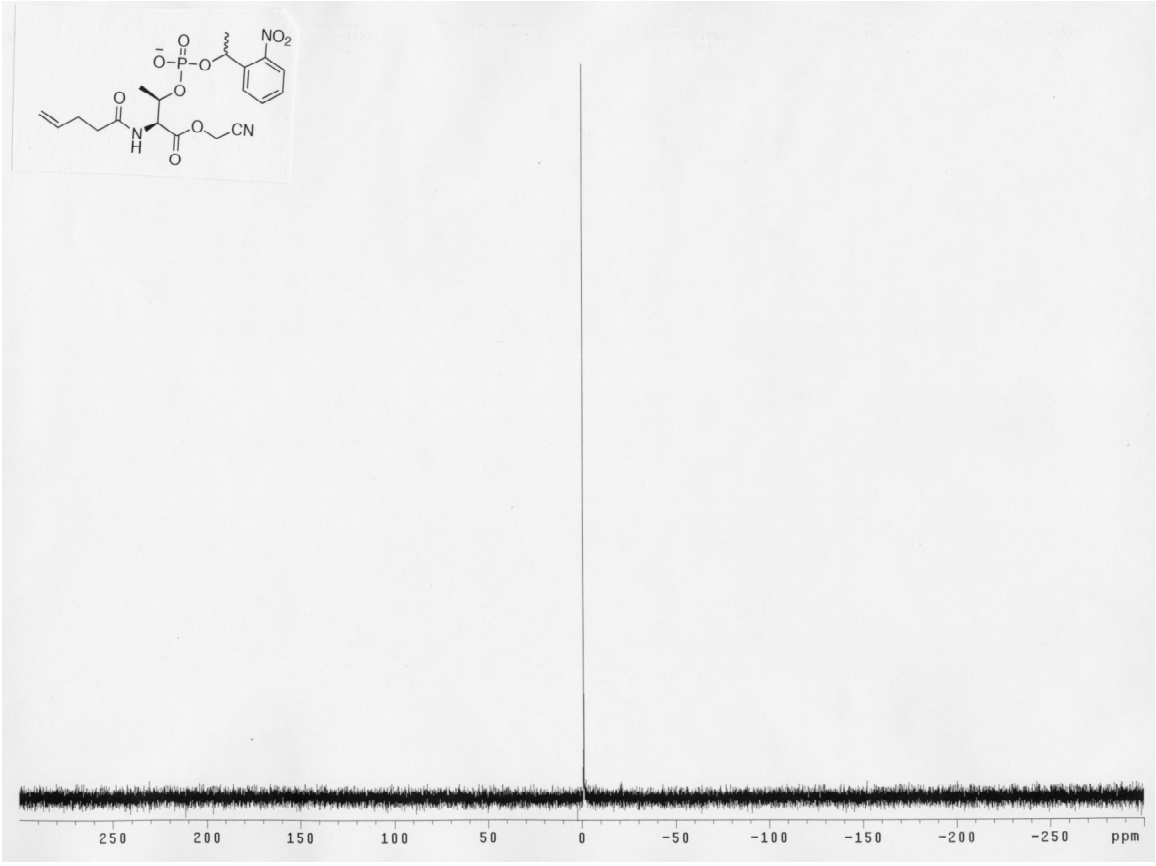




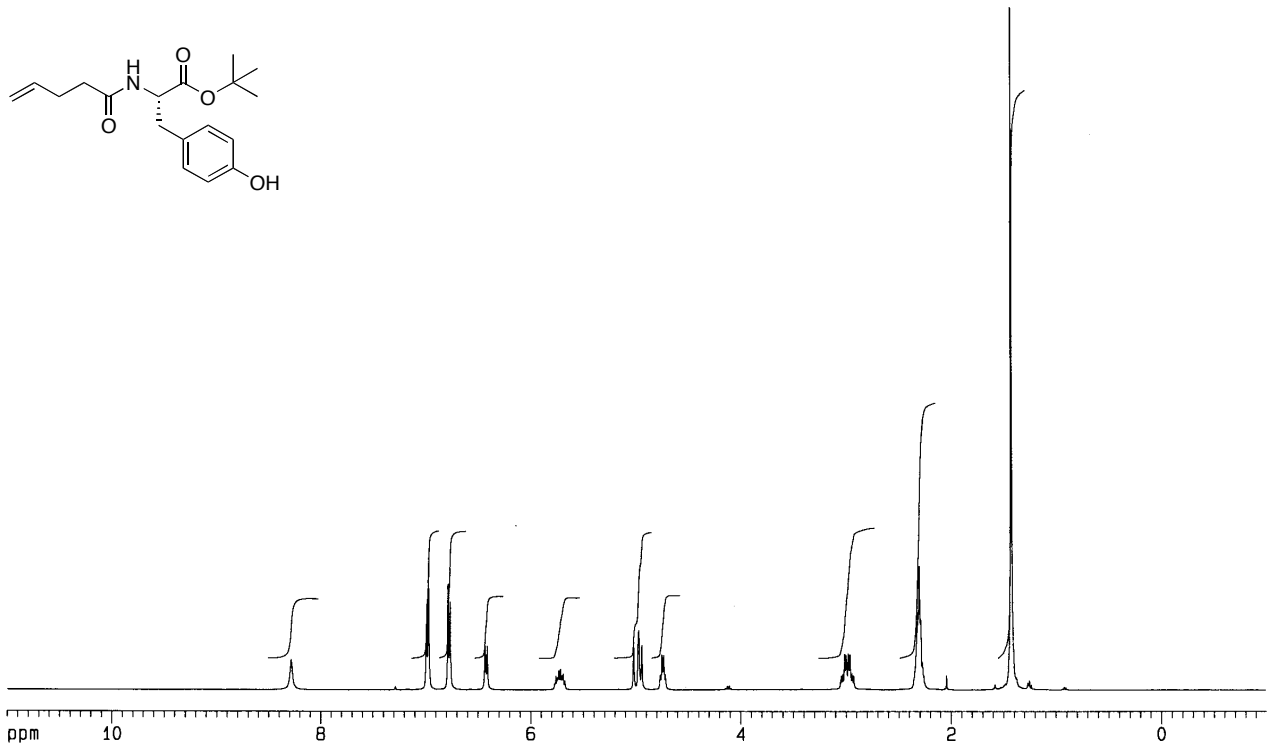
N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl)-L-Threonine cyanomethyl ester (5b)

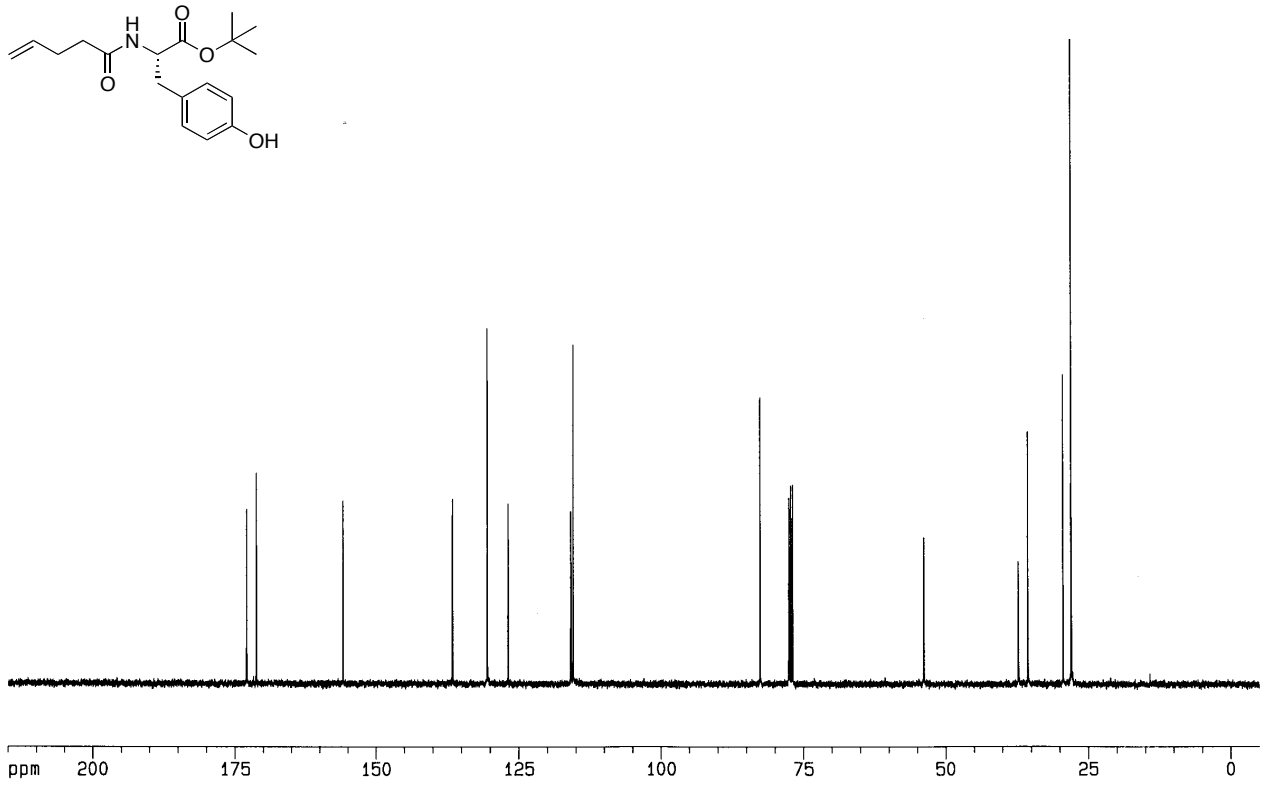




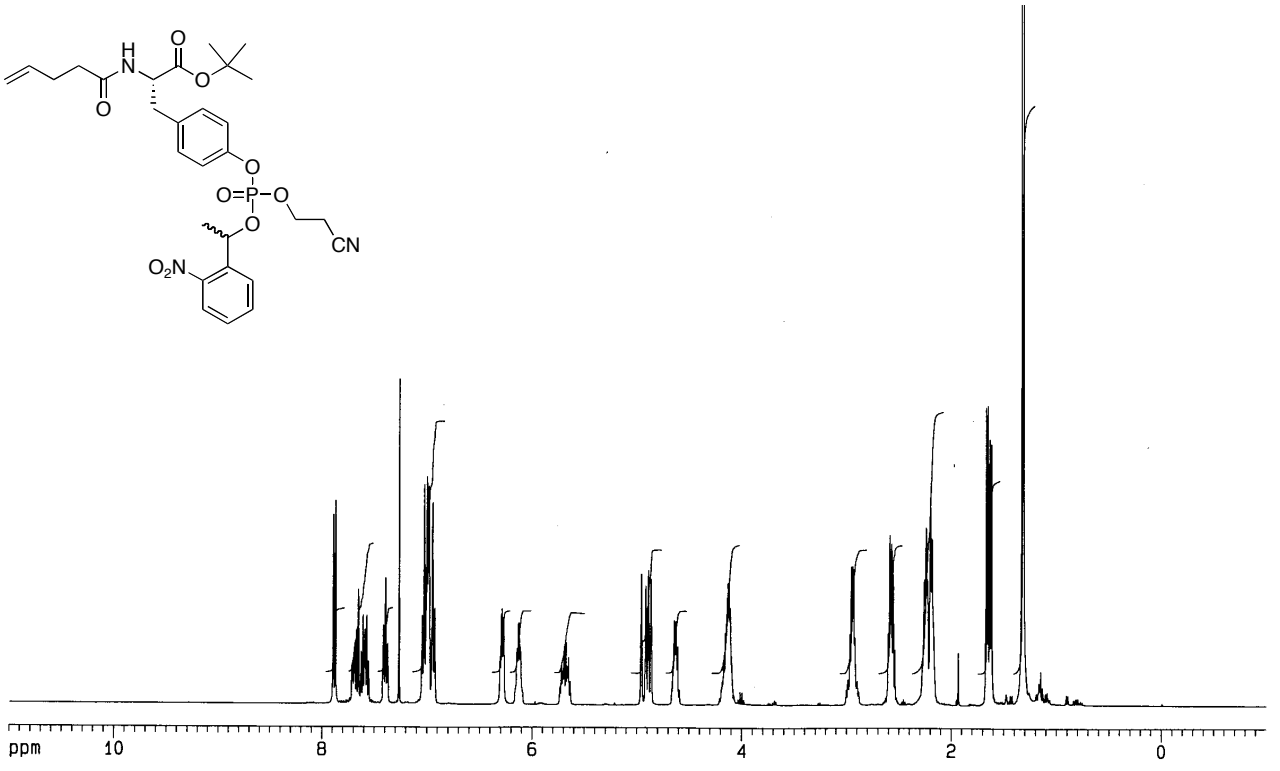

NO2
O
O
P
O
O
O
N
H
O
O
CN
250
200
150
100
50
0
-50
-100
-150
-200
-250
ppm

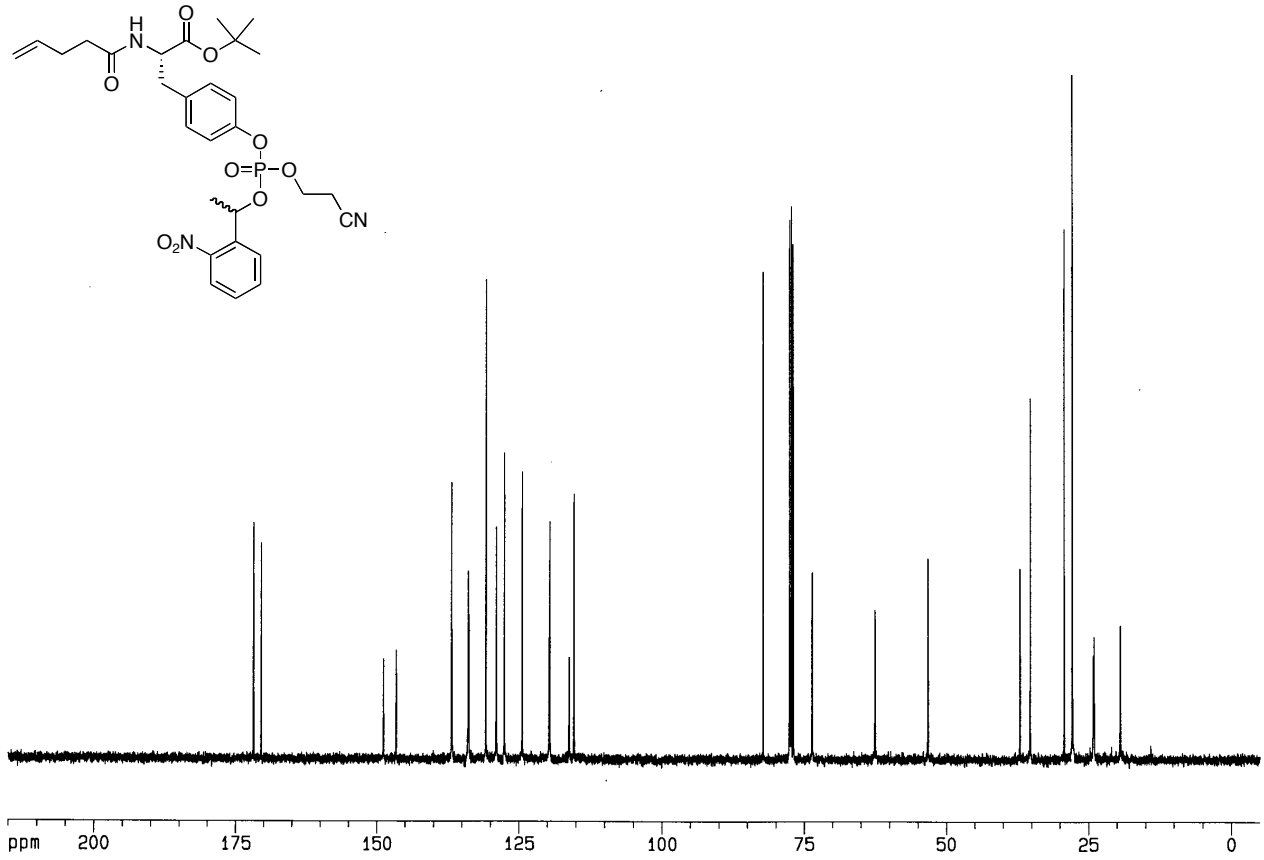
N^{α}-4-pentenoyl-L-Tyrosine *tert*-butyl ester



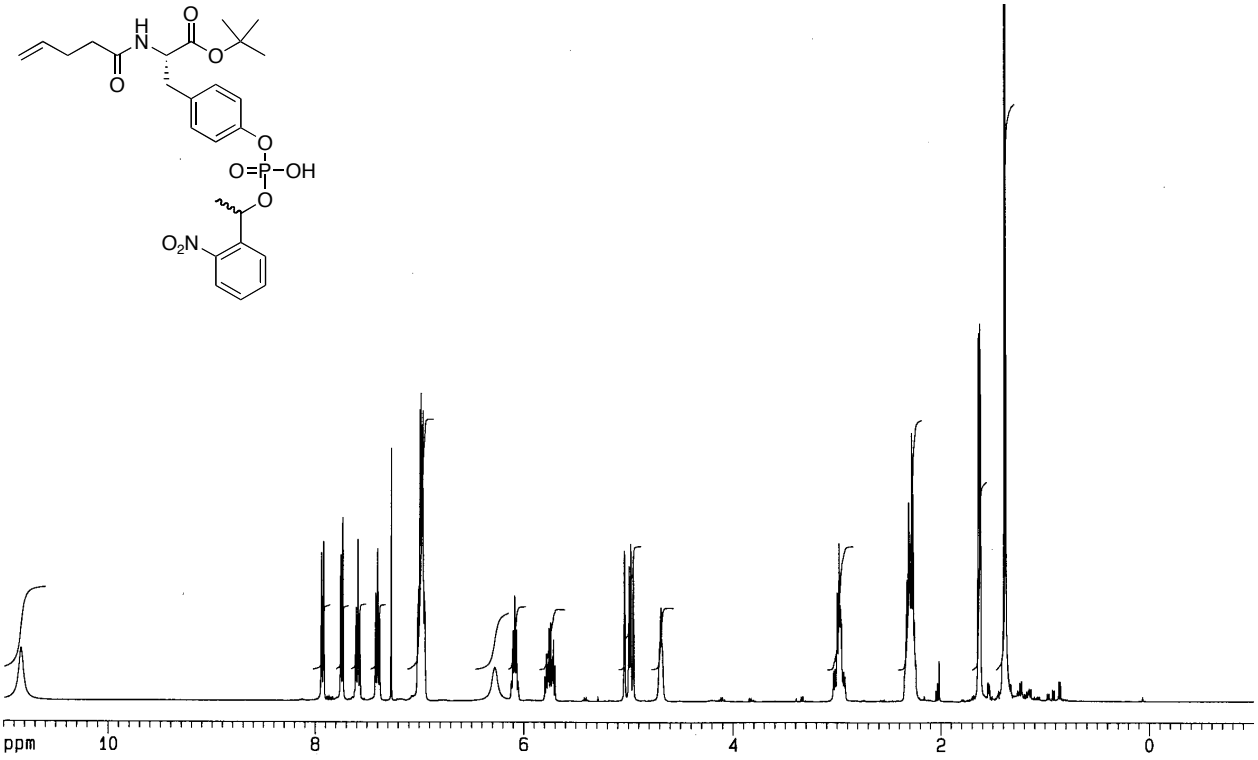


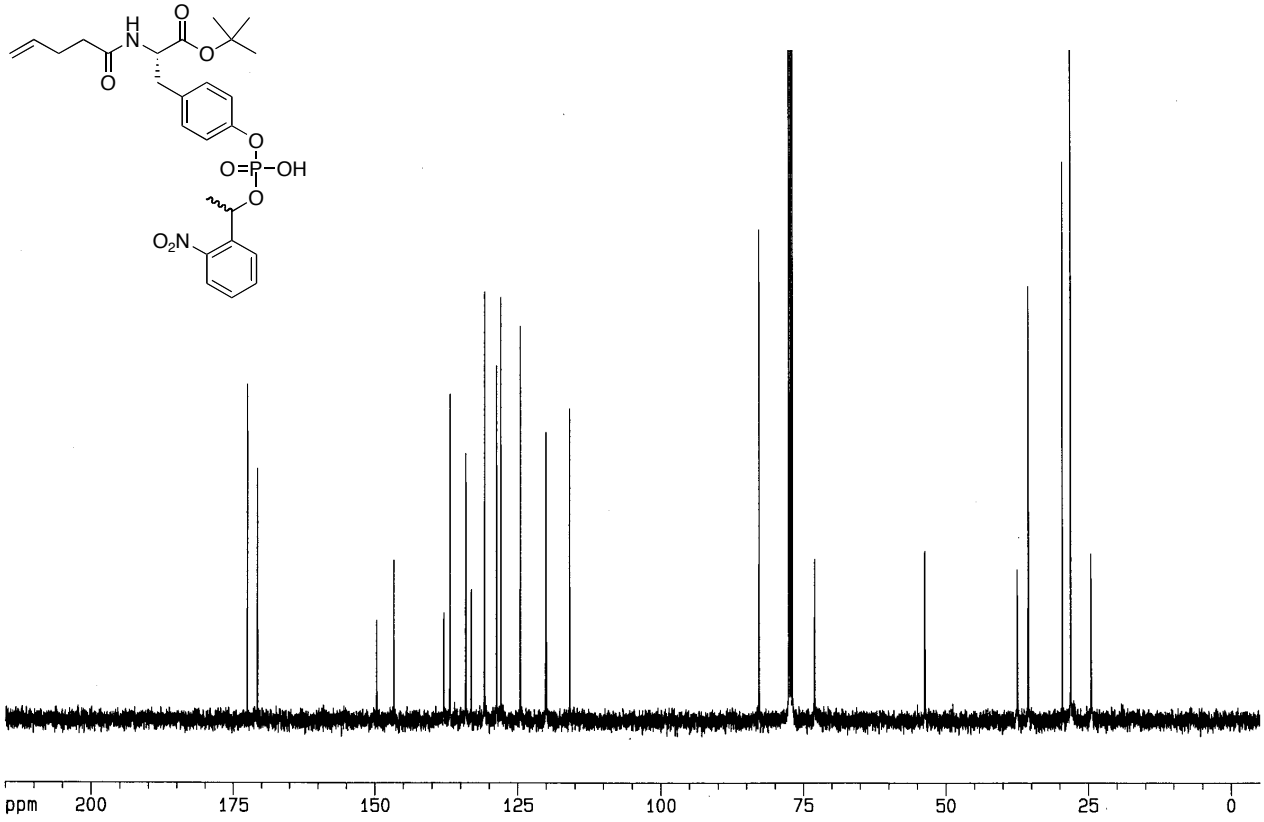
N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-Tyrosine *tert*-butyl ester



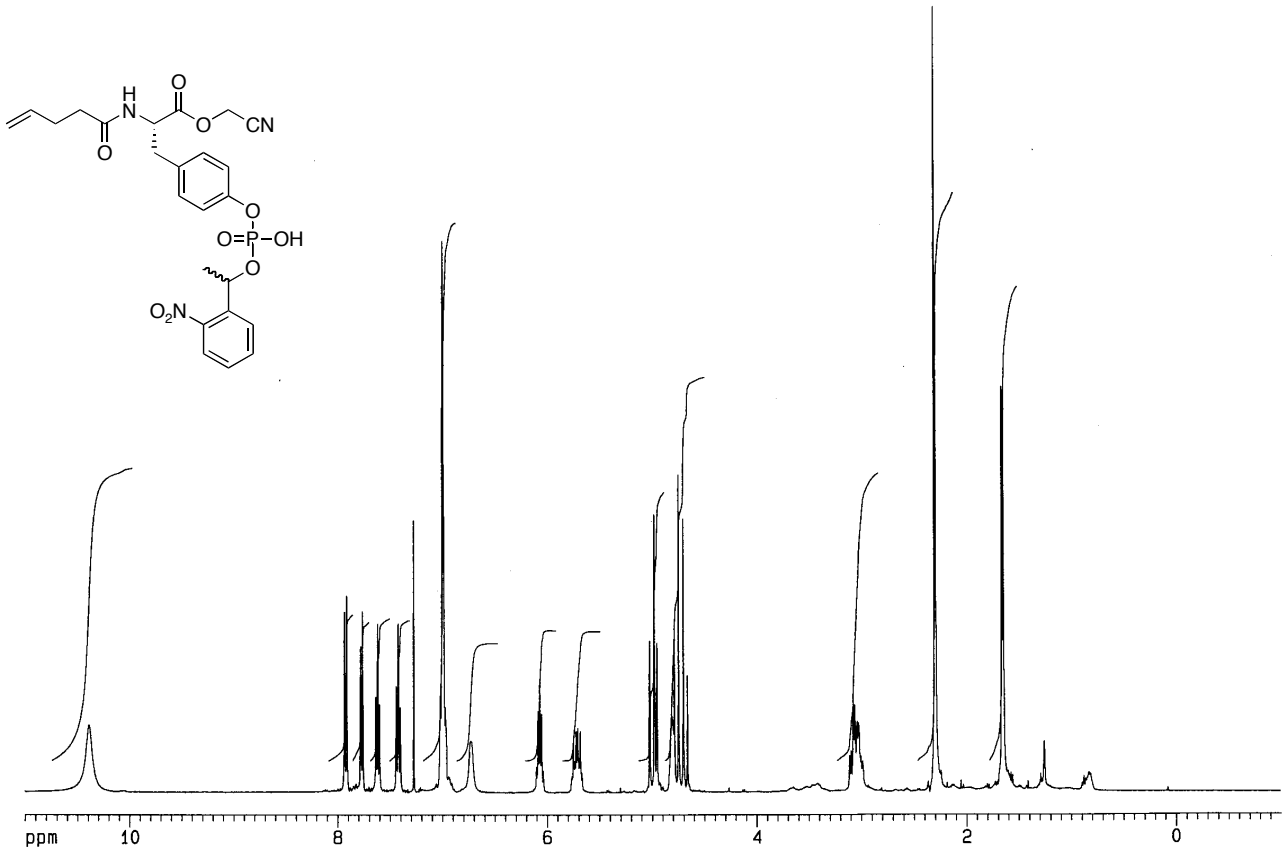


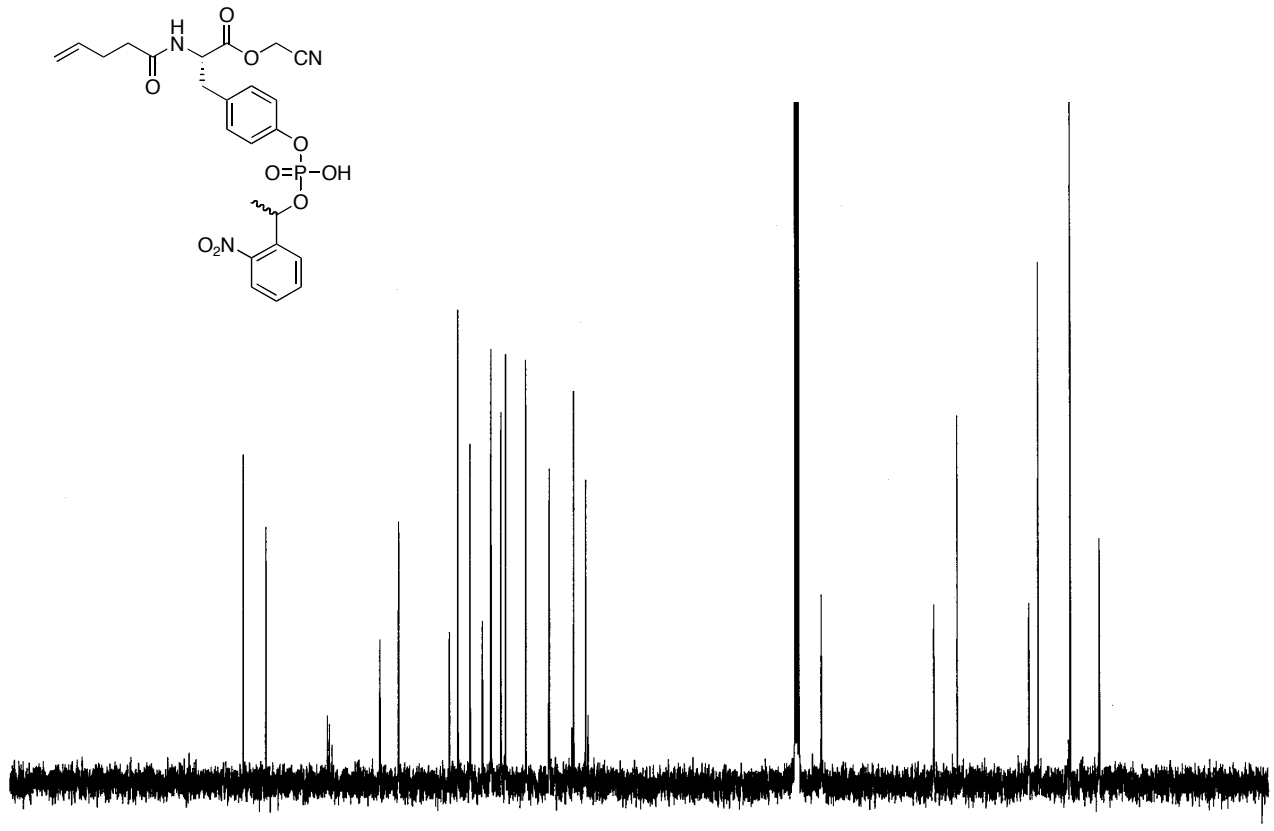
N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl)-L-Tyrosine *tert*-butyl ester





N^{α}-4-pentenoyl-phospho(1-nitrophenylethyl)-L-Tyrosine cyanomethyl ester (6)





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